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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☒ *Additional inventors are being named on the 1 separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

MODIFIED HUMAN GROWTH HORMONE POLYPEPTIDES AND THEIR USES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number **20350**

OR

☐ Firm *or* Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 86 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☐ Other (specify)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: .

Respectfully submitted,

SIGNATURE

Date February 2, 2004

REGISTRATION NO. 47,651 (*if appropriate*)

TYPED or PRINTED NAME Matthew E. Hinsch

Docket Number: 021862-000400US

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

60132175 v1

PROVISIONAL APPLICATION COVER SHEET

Additional Page

PTO/SB/16 (08-03)

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60132175 v1

Attorney Docket No.: 021862-000400US

PATENT APPLICATION

Modified Human Growth Hormone Polypeptides and Their Uses

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Modified Human Growth Hormone Polypeptides and Their Uses

FIELD OF THE INVENTION

This invention relates to growth hormone polypeptides modified with at least one non-naturally-encoded amino acid.

BACKGROUND OF THE INVENTION

[01] The growth hormone (GH) supergene family (Bazan, F. *Immunology Today* 11: 350-354 (1991); Mott, H. R. and Campbell, I. D. *Current Opinion in Structural Biology* 5: 114-121 (1995); Silvennoinen, O. and Ihle, J. N. (1996) SIGNALLING BY THE HEMATOPOIETIC CYTOKINE RECEPTORS) represents a set of proteins with similar structural characteristics. While there are still more members of the family yet to be identified, some members of the family include the following: growth hormone, prolactin, placental lactogen, erythropoietin (EPO), thrombopoietin (TPO), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and cardiotrophin-1 (CT-1) ("the GH supergene family"). Members of the GH supergene family have similar secondary and tertiary structures, despite the fact that they generally have limited amino acid or DNA sequence identity. The shared structural features allow new members of the gene family to be readily identified.

[02] One member of the GH supergene family is human growth hormone (hGH). Human growth hormone participates in much of the regulation of normal human growth and development. This naturally-occurring single-chain pituitary hormone consists of 191 amino acid residues and has molecular weight of approximately 22 kDa. hGH exhibits a multitude of biological effects, including linear growth (somatogenesis), lactation, activation of macrophages, and insulin-like and diabetogenic effects, among others (Chawla, R., *et al.*, *Ann. Rev. Med.* 34:519-547 (1983); Isaksson, O., *et al.*, *Ann. Rev. Physiol.*, 47:483-499 (1985); Hughes, J. and Friesen, H., *Ann. Rev. Physiol.*, 47:469-482 (1985)).

[03] The structure of hGH is well known (Goeddel, D., *et al.*, *Nature* 281:544-548 (1979)), and the three-dimensional structure of hGH has been solved by x-ray crystallography (de Vos, A., *et al.*, *Science* 255:306-312 (1992)). The protein has a compact globular structure, comprising four amphipathic alpha helical bundles, termed A-D beginning from the N-terminus, which are joined by loops. hGH also contains four cysteine residues, which participate in two intramolecular disulfide bonds: C53 is paired with C165 and C182 is paired with C189. The hormone is not glycosylated and has been expressed in a secreted form in *E. coli* (Chang, C., *et al.*, *Gene* 55:189-196 (1987)).

[04] A number of naturally occurring mutants of hGH have been identified. These include hGH-V (Seeberg, *DNA* 1: 239 (1982); U.S. Patent. Nos. 4,446,235, 4,670,393, and 4,665,180) and a 20-kDa hGH containing a deletion of residues 32-46 of hGH (Kostyo *et al.*, *Biochem. Biophys. Acta* 925: 314 (1987); Lewis, U., *et al.*, *J. Biol. Chem.*, 253:2679-2687 (1978)). In addition, numerous hGH variants, arising from post-transcriptional, post-translational, secretory, metabolic processing, and other physiological processes, have been reported (Baumann, G., *Endocrine Reviews* 12: 424 (1991)).

[05] The biological effects of hGH derive from its interaction with specific cellular receptors. The hormone is a member of a family of homologous proteins that include placental lactogens and prolactins. hGH is unusual among the family members, however, in that it exhibits broad species specificity and binds to either the cloned somatogenic (Leung, D., *et al.*, *Nature* 330:537-543 (1987)) or prolactin (Boutin, J., *et al.*, *Cell* 53:69-77 (1988)) receptor. Based on structural and biochemical studies, functional maps for the lactogenic and somatogenic binding domains have been proposed (Cunningham, B. and Wells, J., Proc. Natl. Acad. Sci. 88: 3407 (1991)). The interaction between hGH and extracellular domain of its receptor (hGHbp) is among the most well understood hormone-receptor interactions. High-resolution x-ray crystallographic data (Cunningham, B., *et al.*, *Science*, 254:821-825 (1991)) has shown that hGH has two receptor binding sites and binds two receptor molecules sequentially using distinct sites on the molecule. The two receptor binding sites are referred to as Site I and Site II. Site I includes the carboxy terminal end of helix D and parts of helix A and the A-B loop, whereas Site II encompasses the amino terminal region of helix A and a portion of helix C. Binding of GH to its receptor occurs sequentially, with site I binding first. Site II then engages a second GH receptor, resulting in receptor dimerization and activation of the intracellular signaling pathways that lead to cellular responses to the hormone. An hGH mutein in which a G120R substitution has been introduced into site II is able to bind a single hGH receptor, but is unable to dimerize two receptors. The mutein acts as an hGH

antagonist *in vitro*, presumably by occupying receptor sites without activating intracellular signaling pathways (Fuh, G., *et al.*, *Science* 256:1677-1680 (1992)).

[06] Recombinant hGH is used as a therapeutic and has been approved for the treatment of a number of indications. hGH deficiency leads to dwarfism, for example, which has been successfully treated for more than a decade by exogenous administration of the hormone. In addition to hGH deficiency, hGH has also been approved for the treatment of renal failure (in children), Turner's Syndrome, and cachexia in AIDS patients. Recently, the Food and Drug Administration (FDA) has approved hGH for the treatment of non-GH-dependent short stature. hGH is also currently under investigation for the treatment of aging, frailty in the elderly, short bowel syndrome and congestive heart failure.

[07] Recombinant hGH is currently sold as a daily injectable product, with five major products currently on the market: Humatrope™ (Eli Lilly & Co.), Nutropin™ (Genentech), Norditropin™ (Novo-Nordisk), Genotropin™ (Pfizer) and Saizen/Serostim™ (Serono). A significant challenge to using growth hormone as a therapeutic, however, is that the protein has a short *in vivo* half-life and, therefore, it must be administered by daily subcutaneous injection for maximum effectiveness (MacGillivray, *et al.*, *J. Clin. Endocrinol. Metab.* 81: 1806-1809 (1996)). Considerable effort is focused on means to improve the administration of hGH agonists and antagonists, by lowering the cost of production, making administration easier for the patient, improving efficacy and safety profile, and creating other properties that would provide a competitive advantage. For example, Genentech markets Nutropin Depot™, which is a depot formulation of hGH. While the depot permits less frequent administration (once every 2-3 weeks rather than once daily), it is also associated with undesirable side effects, such as decreased bioavailability and pain at the injection site. Another product, Pegvisomant™ (Pfizer), has also recently been approved by the FDA. Pegvisomant™ is a genetically-engineered analogue of hGH that functions as a highly selective growth hormone receptor antagonist indicated for the treatment of acromegaly (van der Lely, *et al.*, *The Lancet* 358: 1754-1759 (2001). Although several of the amino acid side chain residues in Pegvisomant™ are derivatized with polyethylene glycol (PEG) polymers, the product is still administered once-daily, indicating that the pharmaceutical properties are not optimal. In addition to PEGylation and depot formulations, other administration routes, including inhaled and oral dosage forms of hGH, are under early-stage pre-clinical and clinical development and none has yet received approval from the FDA. Accordingly, there is a need for a polypeptide that exhibits growth hormone activity but that also provides a longer serum half-life and, therefore, more optimal therapeutic levels of hGH.

[08] The present invention addresses, among other things, problems associated with the production of an hGH polypeptide with a longer serum half-life.

BRIEF SUMMARY OF THE INVENTION

[09] This invention provides GH supergene family members, including hGH polypeptides, comprising a non-naturally encoded amino acid.

[10] In some embodiments, the hGH polypeptide is linked to a second hGH polypeptide.

[11] In some embodiments, the non-naturally encoded amino acid is linked to a water soluble polymer. In some embodiments, the water soluble polymer comprises a poly(ethylene glycol) moiety. In some embodiments, the poly(ethylene glycol) molecule is a bifunctional polymer. In some embodiments, the bifunctional polymer is linked to a second polypeptide. In some embodiments, the second polypeptide is an hGH polypeptide.

[12] In some embodiments, the hGH polypeptide comprises at least two amino acids linked to a water soluble polymer comprising a poly(ethylene glycol) moiety. In some embodiments, at least one amino acid is a non-naturally encoded amino acid.

[13] In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1-5, 32-46, 97-105, 132-149, and 184-191 from SEQ ID NO: 2. In some embodiments, the non-naturally encoded amino acid is at a position selected from the group consisting of residues 1, 2, 3, 4, 5, 9, 11, 12, 15, 16, 19, 22, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,43, 44, 45, 46, 47, 48, 49, 52, 55, 57, 59, 65, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 109, 111, 112, 113, 115, 116, 119, 120, 122, 123, 126, 127, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 158, 159, 161, 168, 172, 183, 184, 185, 186, 187, 188, 189, 190, and 191 from SEQ ID NO: 2.

[14] In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 29, 30, 33, 34, 35, 37, 39, 40, 49, 57, 59, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 107, 108, 111, 122, 126, 129, 130, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 159, 183, 186, and 187, and a combination thereof from SEQ ID NO: 2.

[15] In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 33, 35, 37, 57, 69, 70,

71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 129, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 186, and 187, and a combination thereof from SEQ ID NO: 2.

[16] In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155, and a combination thereof from SEQ ID NO: 2.

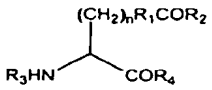
[17] In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of 30, 74, 103, and a combination thereof, from SEQ ID NO: 2.

[18] In some embodiments, the hGH polypeptide comprises a substitution, addition or deletion that increases affinity of the hGH polypeptide for a growth hormone receptor. In some embodiments, the hGH polypeptide comprises a substitution, addition, or deletion that increases the stability of the hGH polypeptide. In some embodiments, the hGH polypeptide comprises an amino acid substitution selected from the group consisting of F10A, F10H, F10I; M14W, M14Q, M14G; H18D; H21N; R167N; D171S; E174S; F176Y, I179T and combination thereof in SEQ ID NO: 2.

[19] In some embodiments, the non-naturally encoded amino acid is reactive toward a water soluble polymer that is otherwise unreactive toward any of the 20 common amino acids.

[20] In some embodiments, the non-naturally encoded amino acid comprises a carbonyl group, an aminooxy group, a hydrazine group, a hydrazide group, a semicarbazide group, an azide group, or an alkyne group.

[21] In some embodiments, the non-naturally encoded amino acid comprises a carbonyl group. In some embodiments, the non-naturally encoded amino acid has the structure:

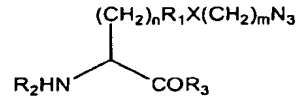


wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, or substituted aryl; R_2 is H, an alkyl, aryl, substituted alkyl, and substituted aryl; and R_3 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_4 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

[22] In some embodiments, the non-naturally encoded amino acid comprises an aminooxy group. In some embodiments, the non-naturally encoded amino acid

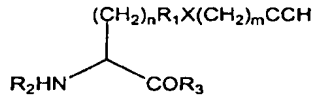
comprises a hydrazide group. In some embodiments, the non-naturally encoded amino acid comprises a hydrazine group. In some embodiments, the non-naturally encoded amino acid residue comprises a semicarbazide group.

[23] In some embodiments, the non-naturally encoded amino acid residue comprises an azide group. In some embodiments, the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, substituted aryl or not present; X is O, N, S or not present; m is 0-10; R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

[24] In some embodiments, the non-naturally encoded amino acid comprises an alkyne group. In some embodiments, the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R1 is an alkyl, aryl, substituted alkyl, or substituted aryl; X is O, N, S or not present; m is 0-10, R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group..

[25] In some embodiments, the poly(ethylene glycol) molecule has a molecular weight of between about 1 and about 50 kDa. In some embodiments, the poly(ethylene glycol) molecule has a molecular weight of between 10 kDa and 40 kDa.

[26] In some embodiments, the hGH polypeptide linked to the water soluble polymer is made by reacting an hGH polypeptide comprising a carbonyl-containing amino acid with a poly(ethylene glycol) molecule comprising a hydroxylamine, hydrazine, hydrazide or semicarbazide group. In some embodiments, the hydroxylamine, hydrazine, hydrazide or semicarbazide group is linked to the poly(ethylene glycol) molecule through an amide linkage.

[27] In some embodiments, the hGH polypeptide linked to the water soluble polymer is made by reacting a poly(ethylene glycol) molecule comprising a carbonyl group

with a polypeptide comprising a non-naturally encoded amino acid that comprises a hydroxylamine, hydrazide or semicarbazide group.

[28] In some embodiments, the hGH polypeptide linked to the water soluble polymer is made by reacting an hGH polypeptide comprising an alkyne-containing amino acid with a poly(ethylene glycol) molecule comprising an azide moiety. In some embodiments, the azide or alkyne group is linked to the poly(ethylene glycol) molecule through an amide linkage.

[29] In some embodiments, the hGH polypeptide linked to the water soluble polymer is made by reacting an hGH polypeptide comprising an azide-containing amino acid with a poly(ethylene glycol) molecule comprising an alkyne moiety. In some embodiments, the azide or alkyne group is linked to the poly(ethylene glycol) molecule through an amide linkage.

[30] In some embodiments, the poly(ethylene glycol) molecule is a branched polymer. In some embodiments, each branch of the poly(ethylene glycol) branched polymer has a molecular weight of between 15 kDa and 30 kDa.

[31] In some embodiments, the polypeptide is a growth hormone antagonist. In some embodiments, the growth hormone antagonist comprises a non-naturally encoded amino acid is linked to a water soluble polymer. In some embodiments, the water soluble polymer comprises a poly(ethylene glycol) moiety. In some embodiments, the non-naturally encoded amino acid linked to a water soluble polymer is present within the Site II region (the region of the protein encompassing the amino terminal region of helix A and a portion of helix C) of hGH. In some embodiments, the non-naturally encoded amino acid linked to a water soluble polymer prevents dimerization of the hGH receptor by preventing the hGH antagonist from binding to a second hGH receptor molecule. In some embodiments, an amino acid other than glycine is substituted for G120 in SEQ ID NO: 2. In some embodiments, arginine is substituted for G120 in SEQ ID NO: 2. In some embodiments, a non-naturally encoded amino acid is substituted for G120 in SEQ ID NO: 2.

[32] The present invention also provides isolated nucleic acids comprising a polynucleotide that hybridizes under stringent conditions to SEQ ID NO:1, wherein the polynucleotide comprises at least one selector codon and the polynucleotide encodes an hGH polypeptide comprising a non-naturally encoded amino acid. In some embodiments, the selector codon is selected from the group consisting of an amber codon, ochre codon, opal codon, and a four-base codon.

[33] The present invention also provides methods of making an hGH polypeptide linked to a water soluble polymer. In some embodiments, the method comprises contacting an isolated hGH polypeptide comprising a non-naturally encoded amino acid with a water soluble polymer comprising a moiety that reacts with the non-naturally encoded amino acid.

[34] In some embodiments, the water soluble polymer comprises a polyethylene glycol moiety. In some embodiments, the non-naturally encoded amino acid residue comprises a carbonyl group, an aminooxy group, a hydrazide group, a semicarbazide group, an azide group, or an alkyne group. In some embodiments, the non-naturally encoded amino acid residue comprises a carbonyl moiety and the water soluble polymer comprises a hydroxylamine, hydrazide or semicarbazide moiety. In some embodiments, the non-naturally encoded amino acid residue comprises an alkyne moiety and the water soluble polymer comprises an azide moiety. In some embodiments, the non-naturally encoded amino acid residue comprises an azide moiety and the water soluble polymer comprises an alkyne moiety.

[35] In some embodiments, the polyethylene glycol moiety has an average molecular weight of between about 1 and about 50 kDa. In some embodiments, the polyethylene glycol moiety is a branched polymer.

[36] The present invention also provides compositions comprising a hGH polypeptide comprising a non-naturally-encoded amino acid and a pharmaceutically acceptable carrier. In some embodiments, the non-naturally encoded amino acid is linked to a water soluble polymer.

[37] The present invention also provides cells comprising a polynucleotide encoding the hGH polypeptide comprising a non-naturally encoded amino acid. In some embodiments, the cells comprise an orthogonal RNA synthetase and orthogonal tRNA for substituting the non-naturally encoded amino acid into the hGH polypeptide.

[38] The present invention also provides methods of making an hGH polypeptide comprising a non-naturally encoded amino acid. In some embodiments, the methods comprise culturing cells comprising a polynucleotide or polynucleotides encoding an hGH polypeptide, an orthogonal RNA synthetase and an orthogonal tRNA under conditions to permit expression of the hGH polypeptide; and purifying the hGH polypeptide from the cells.

[39] The present invention also provides methods of increasing serum half-life or circulation time of hGH. In some embodiments, the methods comprise substituting a

non-naturally encoded mino acid for any one or more amino acids in naturally occurring hGH.

[40] The present invention also provides methods of treating a patient having a growth or development disorder. In some embodiments, the methods comprise administering to the patient a therapeutically-effective amount of a pharmaceutical composition comprising an hGH polypeptide comprising a non-naturally-encoded amino acid and a pharmaceutically acceptable carrier.

[41] The present invention also provides hGH polypeptides comprising a sequence shown in SEQ ID NO: 3, except that at least one amino acid is substituted by a non-naturally encoded amino acid. In some embodiments, the non-naturally encoded amino acid is linked to a water soluble polymer. In some embodiments, the water soluble polymer comprises a poly(ethylene glycol) moiety. In some embodiments, the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1-5, 82-90, 117-134, and 169-176 from SEQ ID NO: 3. In some embodiments, the non-naturally encoded amino acid comprises a carbonyl group, an aminooxy group, a hydrazide group, a hydrazine group, a semicarbazide group, an azide group, or an alkyne group.

[42] In some embodiments, the poly(ethylene glycol) moiety has a molecular weight of between about 1 and about 50 kDa. In some embodiments, the poly(ethylene glycol) moiety has a molecular weight of between 5 kDa and 40 kDa. In some embodiments, the polyethylene glycol moiety is a branched polymer.

[43] The present invention also provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and an hGH polypeptide comprising the sequence shown in SEQ ID NO: 3, wherein at least one amine acid is substituted by a non-naturally encoded amino acid. In some embodiments, the non-naturally encoded amino acid comprises a saccharide moiety. In some embodiments, the water soluble polymer is linked to the polypeptide via a saccharide moiety.

DEFINITIONS

[44] The term "human Growth Hormone (hGH)" or "hGH polypeptide" refers to a polypeptide that retains at least one biological activity of naturally-occurring hGH. hGH polypeptides include the pharmaceutically acceptable salts and prodrugs, and prodrugs of the salts, polymorphs, hydrates, solvates, biologically-active fragments, biologically-active variants and stereoisomers of the naturally-occurring human Growth Hormone as well as agonist and antagonist variants of the naturally-occurring human Growth Hormone and

polypeptide fusions thereof. Fusions comprising additional amino acids at the amino terminus, carboxyl terminus, or both, are encompassed by the term "hGH polypeptide." Exemplary fusions include, but are not limited to, e.g., methionyl growth hormone in which a methionine is linked to the N-terminus of hGH, fusions for the purpose of purification (e.g., to poly-histadine or affinity epitopes), fusions with serum albumin binding peptides and fusions with serum proteins such as serum albumin. The naturally-occurring hGH nucleic acid and amino acid sequences are known. *See, e.g.*, Goeddel, D., *et al.*, *Nature* 281:544-548 (1979); Gray, G., *et al.*, *Gene*, 39:247-254 (1985). For the complete naturally-occurring hGH amino acid sequence as well as the mature naturally-occurring hGH amino acid sequence, see SEQ ID NO:1 and SEQ ID NO:2, respectively, herein. In some embodiments, hGH polypeptides of the invention are substantially identical to SEQ ID NO:2 or SEQ ID NO:3.

[45] Biologically-active fragments/variants of hGH include the 20-kDa hGH variant in which residues 32-46 of hGH are deleted (SEQ ID NO:3) (*see, e.g.*, Kostyo *et al.*, *Biochem. Biophys. Acta* 925: 314 (1987); Lewis, U., *et al.*, *J. Biol. Chem.*, 253:2679-2687 (1978)) as well as placental growth hormone (Igout A., *et al.*, *Nucleic Acids Res.* 17(10):3998 (1989)). The term "hGH polypeptide" also includes proteolytically cleaved or two-chain variants of GH (Baumann, G., *Endocrine Reviews*, 12:424-449 (1991)) and hGH dimers linked directly via Cys-Cys disulfide linkages (Lewis, U. J., *et al.*, *J. Biol. Chem.* 252: 3697-3702 (1977); Brostedt, P. and Roos, P., *Prep. Biochem.* 19: 217-229 (1989)).

[46] All references to amino acid positions in hGH described herein are based on the position in SEQ ID NO:2, unless otherwise specified (i.e., when it is stated that the comparison is based on SEQ ID NO:3). Those of skill in the art will appreciate that amino acid positions corresponding to positions in SEQ ID NO:2 can be readily identified in hGH fusions, variants, fragments, etc. For example, sequence alignment programs such as BLAST can be used to align and identify a particular position in a protein that corresponds with a position inSEQ ID NO:2. Substitutions, deletions or additions of amino acids described herein in reference to SEQ ID NO:2 are intended to also refer to substitutions, deletions or additions in corresponding positions in hGH fusions, variants, fragments, etc. described herein or known in the art and are expressly encompassed by the present invention.

[47] The term "hGH polypeptide" encompasses hGH polypeptides comprising one or more amino acid substitutions, additions or deletions. Exemplary substitutions include, e.g., substitution of the lysine at position 41 or the phenylalanine at position 176 of native hGH. In some cases, the substitution may be an isoleucine or arginine residue if the substitution is at position 41 or is a tyrosine residue if the position is 176.

Position F10 can be substituted with, e.g., A, H or I. Position M14 may be substituted with, e.g., W, Q or G. Other exemplary substitutions include any substitutions or combinations thereof, including but not limited to:

R167N, D171S, E174S, F176Y, I179T;

R167E, D171S, E174S, F176Y;

F10A, M14W, H18D, H21N;

F10A, M14W, H18D, H21N, R167N, D171S, E174S, F176Y, I179T;

F10A, M14W, H18D, H21N, R167N, D171A, E174S, F176Y, I179T;

F10H, M14G, H18N, H21N; F10A, M14W, H18D, H21N, R167N, D171A T175T, I179T; or F10I M14Q, H18E, R167N, D171S, I179T. *See, e.g.*, U.S. Patent No. 6,143,523.

[48] Excmplary substitutions in a wide variety of amino acid positions in naturally-occurring hGH have been described, including substitutions that increase agonist activity, convert the polypeptide into an antagonist, etc. and are encompassed by the term "hGH polypeptide."

[49] Agonist hGH sequences include, e.g., the naturally-occurring hGH sequence comprising the following modifications H18D, H21N, R167N, D171S, E174S, I179T. *See, e.g.*, U.S. Patent Nos. 5,849,535; and 6,022,711.

[50] Human Growth Hormone antagonists include, e.g., those with a substitution at G120 (e.g., G120R, G120K, G120W, G120Y, G120F, or G120E) and sometimes further including the following substitutions: H18A, Q22A, F25A, D26A, Q29A, E65A, K168A, E174A. *See, e.g.* U.S. Patent No. 6,004,931. In some embodiments, hGH antagonists comprise at least one substitution in the regions 106-108 or 127-129 that cause GH to act as an antagonist. *See, e.g.*, U.S. Patent No. 6,608,183. In some embodiments, the hGH antagonist comprises a non-naturally encoded amino acid linked to a water soluble polymer that is present in the Site II binding region of the hGH molecule. In some embodiments, the hGH polypeptide further comprises the following substitutions: H18D, H21N, R167N, K168A, D171S, K172R, E174S, I179T.

[51] In some embodiments, the hGH polypeptides further comprise an addition, substitution or deletion that modulates biological activity of hGH. For example, the additions, substitutions or deletions may modulate affinity for the hGH receptor, modulate (e.g., increases or decreases) receptor dimerization, stabilize receptor dimers, modulate circulating half-life, modulate release or bio-availaibilty, facilitate purification, or improve or alter a particular route of administration. Similarly, hGH polypeptides can comprise protease cleavage sequences, reactive groups, antibody-binding domains (e.g., FLAG or poly-His) or

other affinity based sequences (e.g., FLAG, poly-His, GST, etc.) or linked molecules (e.g., biotin) that improve detection (e.g., GFP), purification or other traits of the polypeptide.

[52] The term "hGH polypeptide" also encompasses hGH dimers linked directly via non-naturally encoded amino acid side chains, either to the same or different non-naturally encoded amino acid side chains, to naturally-encoded amino acid side chains, or indirectly via a linker. Exemplary linkers include, e.g., water soluble polymers such as poly(ethylene glycol) or polydextran or a polypeptide.

[53] A "non-naturally encoded amino acid" refers to an amino acid that is not one of the 20 common amino acids or pyrolysine or selenocysteine. The term "non-naturally encoded amino acid" includes amino acids that occur naturally by modification of a naturally encoded amino acid (e.g., the 20 common amino acids or pyrolysine and selenocysteine) but are not themselves encoded by a cell. Examples of naturally-occurring amino acids that are not naturally-encoded include, but are not limited to, *N*-acetylglucosaminyl-L-serine, *N*-acetylglucosaminyl-L-threonine, and O-phosphotyrosine.

[54] An "amino terminus modification group" refers to any molecule that can be attached to the amino terminus of a polypeptide. Similarly, a "carboxy terminus modification group" refers to any molecule that can be attached to the carboxy terminus of a polypeptide. Terminus modification groups include but are not limited to various water soluble polymers, peptides or proteins such as serum albumin, or other moieties that increase serum half-life of peptides.

[55] A "bifunctional polymer" refers to a polymer comprising two discrete functional groups that are capable of reacting specifically with other moieties (e.g., amino acid side groups) to form covalent or non-covalent linkages. A bifunctional linker having one functional group reactive with a group on a particular biologically active component, and another group reactive with a group on a second biological component, may be used to form a conjugate that includes the first biologically active component, the bifunctional linker and the second biologically active component. Many procedures and linker molecules for attachment of various compounds to peptides are known. *See, e.g.*, European Patent Application No. 188,256; U.S. Patent Nos.4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; 4,569,789; and 4,589,071. A "multi-functional polymer" refers to a polymer comprising two or more discrete functional groups that are capable of reacting specifically with other moieties (e.g., amino acid side groups) to form covalent or non-covalent linkages.

[56] Where substituent groups are specified by their conventional chemical formulas, written from left to right, they equally encompass the chemically identical

substituents that would result from writing the structure from right to left, *e.g.*, $-CH_2O-$ is equivalent to $-OCH_2-$.

[57] The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups which are limited to hydrocarbon groups are termed "homoalkyl".

[58] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by the structures $-CH_2CH_2-$ and $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[59] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[60] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-$

CH_2-O-CH_3, -CH_2-CH_2-NH-CH_3, -CH_2-CH_2-N(CH_3)-CH_3, -CH_2-S-CH_2-CH_3, -CH_2-CH_2,-S(O)-CH_3, -CH_2-CH_2-S(O)$_2$-CH_3, -CH=CH-O-CH_3, -Si(CH_3)$_3$, -CH_2-CH=N-OCH_3, and –CH=CH-N(CH_3)-CH_3. Up to two heteroatoms may be consecutive, such as, for example, -CH_2-NH-OCH_3 and –CH_2-O-Si(CH_3)$_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, -CH_2-CH_2-S-CH_2-CH_2- and –CH_2-S-CH_2-CH_2-NH-CH_2-. For heteroalkylene groups, the same or different heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, aminooxyalkylene, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula –C(O)$_2$R'- represents both –C(O)$_2$R'- and –R'C(O)$_2$-.

[61] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Thus, a cycloalkyl or heterocycloalkyl include saturated and unsaturated ring linkages. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1–(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1–piperazinyl, 2-piperazinyl, and the like.

[62] As used herein, the term "water soluble polymer" refers to any polymer that may be linked to an hGH polypeptide *in vivo* or *in vitro* to result in increased solubility of the hGH polypeptide in aqueous solvents. Linkage of water soluble polymers to HGH can result in changes including, but not limited to, increased serum half-life relative to the unmodified form and altered receptor binding. The water soluble polymer may or may not have its own biological activity. Suitable polymers include, for example, polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, polyamino acids, divinylether maleic anhydride, *N*-(2-Hydroxypropyl)-methacrylamide, dextran, dextran derivatives including dextran sulfate, polypropylene glycol, polyoxyethylated polyol, heparin, heparin fragments, polysaccharides, oligosaccharides, glycans, cellulose and cellulose derivatives, including methylcellulose and carboxymethyl cellulose, starch and starch derivatives, polypeptides, polyalkylene glycol and derivatives thereof, copolymers of polyalkylene glycols and

derivatives thereof, polyvinyl ethyl ethers, and alpha-beta-poly[(2-hydroxycthyl)-DL-aspartamide, and the like, or mixtures thereof. Examples of such water soluble polymers include but are not limited to polyethylene glycol and serum albumin.

[63] As used herein, the term "polyalkylene glycol" refers to polyethylene glycol, polypropylene glycol, polybutylene glycol, and derivatives thereof. The term "polyalkylene glycol" encompasses both linear and branched polymers and average molecular weights of between 1 kDa and 100 kDa. Other exemplary embodiments are listed in Shearwater Corporation's catalog "Polyethylene Glycol and Derivatives for Biomedical Applications" (2001).

[64] The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[65] For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[66] Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") are meant to include both substituted and unsubstituted forms of the indicated radical. Exemplary substituents for each type of radical are provided below.

[67] Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', $-NR"C(O)_2R'$, -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', $-S(O)R'$, $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such a radical. R', R", R"' and R"" each independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, *e.g.*, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, $-CF_3$ and $-CH_2CF_3$) and acyl (*e.g.*, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like).

[68] Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are varied and are selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', $-NR"C(O)_2R'$, -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', $-S(O)R'$, $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$, -R', $-N_3$, $-CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are independently selected from hydrogen, alkyl, heteroalkyl, aryl and heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present.

[69] As used herein, the term "increased serum half-life" means the positive change in circulating half-life of a modified biologically active molecule relative to its non-modified form. Serum half-life is measured by taking blood samples at various time points

after administration of the biologically active molecule, and determining the concentration of that molecule in each sample. Correlation of the serum concentration with time allows calculation of the serum half-life. The increase is desirably at least about two-fold, but a smaller increase may be useful, for example where it enables a satisfactory dosing regimen or avoids a toxic effect. In some embodiments, the increase is at least about three-fold, at least about five-fold, or at least about ten-fold.

[70] The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is substantially free of other cellular components with which it is associated in the natural state. It can be in a homogeneous state. Isolated substances can be in either a dry state or in aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames which flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to substantially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

[71] The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Cassol *et al.* (1992); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)).

[72] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is a non-naturally encoded amino acid. As used herein, the terms

encompass amino acid chains of any length, including full length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds.

[73] The term "amino acid" refers to naturally occurring and non-naturally occurring amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally encoded amino acids include the 20 common amin acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrolysine and selenocysteine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[74] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[75] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally

identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[76] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[77] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

[78] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Sequences are "substantially identical" if they have a percentage of amino acid residues or nucleotides that are the same (*i.e.*, about 60% identity, optionally about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence. The identity can exist over a region that is at least about 50 amino acids or nucleotides in length, or over a region that is 75-100 amino acids or nucleotides in length, or, where not specified, across the entire sequence or a polynucleotide or polypeptide.

[79] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[80] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.,* Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

[81] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLAST algorithm is typically performed with the "low complexity" filter turned off.

[82] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[83] The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA).

[84] The phrase "stringent hybridization conditions" refers to conditions of low ionic strength and high temperature as is known in the art. Typically, under stringent conditions a probe will hybridize to its target subsequence in a complex mixture of nucleic acid (*e.g.*, total cellular or library DNA or RNA) but does not hybridize to other sequences in the complex mixture. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60° C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal may be at least two times background, optionally 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5X SSC, and 1% SDS,

incubating at 42°C, or 5X SSC, 1% SDS, incubating at 65°C, with wash in 0.2X SSC, and 0.1% SDS at 65°C. Such washes can be performed for 5, 15, 30, 60, 120, or more minutes.

DETAILED DESCRIPTION

I. Introduction

[85] The present invention provides methods and compositions based on members of the GH supergene family (including but not limited to human growth hormone (hGH)) comprising at least one non-naturally encoded amino acid. Introduction of at least one non-naturally encoded amino acid into GH supergene family member can allow for the application of conjugation chemistries that involve specific chemical reactions, including, but not limited to, with one or more non-naturally encoded amino acids while not reacting with the commonly occurring 20 amino acids present in the GH supergene family member polypeptide. In some embodiments, the GH supergene family member comprising the non-naturally encoded amino acid is linked to a water soluble polymer, such as polyethylene glycol (PEG), via the side chain of the non-naturally encoded amino acid.

II. Growth Hormone Supergene Family

[86] The following proteins include those encoded by genes of the growth hormone (GH) supergene family (Bazan, F., *Immunology Today* 11: 350-354 (1991); Bazan, J. F. *Science* 257: 410-411 (1992); Mott, H. R. and Campbell, I. D., *Current Opinion in Structural Biology* 5: 114-121 (1995); Silvennoinen, O. and Ihle, J. N., SIGNALLING BY THE HEMATOPOIETIC CYTOKINE RECEPTORS (1996)): growth hormone, prolactin, placental lactogen, erythropoietin (EPO), thrombopoietin (TPO), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, garma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and cardiotrophin-1 (CT-1) ("the GH supergene family"). It is anticipated that additional members of this gene family will be identified in the future through gene cloning and sequencing. Members of the GH supergene family have similar secondary and tertiary structures, despite the fact that they generally have limited amino acid or DNA sequence identity. The shared structural features allow new members of the gene family to be readily identified. Given the extent of structural homology among the members

of the GH supergene family, non-naturally encoded amino acids may be incorporated into any members of the GH supergene family using the present invention.

[87] Structures of a number of cytokines, including G-CSF (Hill, C. P., *Proc. Natl. Acad. Sci. USA* 90:5167-5171 (1993)), GM-CSF (Diederichs, K., *et al. Science* 154: 1779-1782 (1991); Walter *et al., J. Mol. Biol.* 224:1075-1085 (1992)), IL-2 (Bazan, J. F. *Science* 257: 410-411 (1992); McKay, D. B. *Science* 257: 412 (1992)), IL-4 (Redfield et al., *Biochemistry* 30: 11029-11035 (1991); Powers *et al., Science* 256:1673-1677 (1992)), and IL-5 (Milburn et al., *Nature* 363: 172-176 (1993)) have been determined by X-ray diffraction and NMR studies and show striking conservation with the GH structure, despite a lack of significant primary sequence homology. EPO is considered to be a member of this family based upon modeling and mutagenesis studies (Boissel *et al., J. Biol. Chem.* 268: 15983-15993 (1993); Wen et al., J. Biol. Chem. 269: 22839-22846 (1994)). A large number of additional cytokines and growth factors including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), thrombopoietin (TPO), oncostatin M, macrophage colony stimulating factor (M-CSF), IL-3, IL-6, IL-7, IL-9, IL-12, IL-13, IL-15, and alpha, beta, omega, tau and gamma interferon belong to this family (reviewed in Mott and Campbell, *Current Opinion in Structural Biology* 5: 114-121 (1995); Silvennoinen and Ihle (1996) SIGNALLING BY THE HEMATOPOIETIC CYTOKINE RECEPTORS). All of the above cytokines and growth factors are now considered to comprise one large gene family, of which hGH is the prototype.

[88] In addition to sharing similar secondary and tertiary structures, members of this family share the property that they must oligomerize cell surface receptors to activate intracellular signaling pathways. Some GH family members, e.g.; GH and EPO, bind a single type of receptor and cause it to form homodimers. Other family members, e.g., IL-2, IL4. and IL-6, bind more than one type of receptor and cause the receptors to form heterodimers or higher order aggregates (Davis et al., (1993) *Science* 260: 1805-1808; Paonessa et al., 1995) EMBO J. 14: 1942-1951; Mott and Campbell, *Current Opinion in Structural Biology* 5: 114-121 (1995)). Mutagenesis studies have shown that, like GH, these other cytokines and growth factors contain multiple receptor binding sites, typically two, and bind their cognate receptors sequentially (Mott and Campbell, *Current Opinion in Structural Biology* 5: 114-121 (1995); Matthews et al., (1996) *Proc. Natl. Acad. Sci. USA* 93: 9471-9476). Like GH, the primary receptor binding sites for these other family members occur primarily in the four alpha helices and the A-B loop. The specific amino acids in the helical bundles that participate in receptor binding differ amongst the family members. Most of the

cell surface receptors that interact with members of the GH supergene family are structurally related and comprise a second large multi-gene family. *See, e.g.* U.S. Patent No. 6,608,183.

[89] A general conclusion reached from mutational studies of various members of the GH supergene family is that the loops joining the alpha helices generally tend to not be involved in receptor binding. In particular the short B-C loop appears to be non-essential for receptor binding in most, if not all, family members. For this reason, the B-C loop may be substituted with non-naturally encoded amino acids as described herein in members of the GH supergene family. The A-B loop, the C-D loop (and D-E loop of interferon/ IL-10-like members of the GH superfamily) may also be substituted with a non-naturally-occurring amino acid. Amino acids proximal to helix A and distal to the final helix also tend not to be involved in receptor binding and also may be sites for introducing non-naturally-occurring amino acids. In some embodiments, a non-naturally encoded amino acid is substituted within the first 1, 2, 3, 4, 5, 6, 7, or more amino acids of the A-B, B-C, C-D or D-E loop. In some embodiments, a non-naturally encoded amino acid is substituted within the last 1, 2, 3, 4, 5, 6, 7, or more amino acids of the A-B, B-C, C-D or D-E loop.

[90] Certain members of the GH family, e.g., EPO, IL-2, IL-3, IL-4, IL-6, G-CSF, GM-CSF, TPO, IL-10, IL-12 p35, IL-13, IL-15 and beta-interferon contain N-linked and O-linked sugars. The glycosylation sites in the proteins occur almost exclusively in the loop regions and not in the alpha helical bundles. Because the loop regions generally are not involved in receptor binding and because they are sites for the covalent attachment of sugar groups, they may be sites for introducing non-naturally-occurring amino acid substitutions into the proteins. Amino acids that comprise the N- and O-linked glycosylation sites in the proteins may be sites for non-naturally-occurring amino acid substitutions because these amino acids are surface-exposed. Therefore, the natural protein can tolerate bulky sugar groups attached to the proteins at these sites and the glycosylation sites tend to be located away from the receptor binding sites.

[91] Additional members of the GH gene family are likely to be discovered in the future. New members of the GH supergene family can be identified through computer-aided secondary and tertiary structure analyses of the predicted protein sequences. Members of the GH supergene family will possess four or five amphipathic helices joined by non-helical amino acids (the loop regions). The proteins may contain a hydrophobic signal sequence at their N-terminus to promote secretion from the cell. Such later discovered members of the GH supergen family also are included within this invention.

[92] Reference to hGH polypeptides in this application is intended to use hGH as an example of a member of the GH supergene family. Thus, it is understood that the modifications and chemistries described herein with reference to hGH can be equally applied to any other members of the GH supergene family, including those specifically listed herein.

III. General Recombinant Nucleic Acid Methods For Use With The Invention

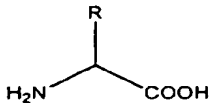
[93] In numerous embodiments of the present invention, nucleic acids encoding a hGH of interest will be isolated, cloned and often altered using recombinant methods. Such embodiments are used, *e.g.*, for protein expression or during the generation of variants, derivatives, expression cassettes, or other sequences derived from a hGH polypeptide In some embodiments, the sequences encoding the polypeptides of the invention are operably linked to a heterologous promoter. Isolation of naturally-occurring hGH is described in, e.g., U.S. Patent Nos. 5,849,535; 6,004,931; 6,022,711; 6,143,523 and 6,608,183. A nucleotide sequence encoding an hGH polypeptide comprising a non-naturally encoded amino acid may be synthesized on the basis of the amino acid sequence of the parent polypeptide, e.g., having the amino acid sequence shown in SEQ ID NO: 2, and then changing the nucleotide sequence so as to effect introduction (i.e., insertion or substitution) or removal (i.e., deletion or substitution) of the relevant amino acid residue(s). The nucleotide sequence may be conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence may be prepared by chemical synthesis, e.g., by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction. *See, e.g.,* Barany, *et al., Proc. Natl. Acad. Sci.* 88: 189-193 (1991); U.S. 6,521,427.

[94] This invention utilizes routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al., Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

IV. Non-Naturally Encoded Amino Acids

[95] Any number of non-naturally encoded amino acids can be introduced into hGH. In general, the introduced non-naturally encoded amino acids are substantially chemically inert toward the 20 common, genetically-encoded amino acids (i.e., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine). In some embodiments, the non-naturally encoded amino acids include side chain functional groups that react efficiently and selectively with functional groups not found in the 20 common amino acids (e.g., azido, ketone, aldehyde and aminooxy groups) to form stable conjugates. For example, an hGH polypeptide that includes a non-naturally encoded amino acid containing an azido functional group can be reacted with a polymer (e.g., poly(ethylene glycol) or, alternatively, a second polypeptide containing an alkyne moiety to form a stable conjugate resulting for the selective reaction of the azide and the alkyne functional groups to form a Huisgen [3+2] cycloaddition product.

[96] The generic structure of an alpha-amino acid is illustrated as follows:



[97] A non-naturally encoded amino acid is typically any structure having the above-listed formula wherein the R group is any substituent other than one used in the twenty natural amino acids. Because the non-naturally encoded amino acids of the invention typically differ from the natural amino acids only in the structure of the side chain, the non-naturally encoded amino acids form amide bonds with other amino acids, e.g., natural or non-naturally encoded, in the same manner in which they are formed in naturally occurring polypeptides. However, the non-naturally encoded amino acids have side chain groups that distinguish them from the natural amino acids. For example, R optionally comprises an alkyl-, aryl-, acyl-, keto-, azido-, hydroxyl-, hydrazine, cyano-, halo-, hydrazide, alkenyl, alkynl, ether, thiol, seleno-, sulfonyl-, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, ester, thioacid, hydroxylamine, amino group, or the like or any combination thereof. Other non-naturally occurring amino acids of interest include, but are not limited to, amino acids comprising a photoactivatable cross-linker, spin-labeled amino acids, fluorescent amino acids, metal binding amino acids, metal-containing amino acids, radioactive amino acids, amino acids with novel functional groups, amino acids that covalently or noncovalently interact with other molecules, photocaged and/or

photoisomerizable amino acids, amino acids comprising biotin or a biotin analogue, glycosylated amino acids such as a sugar substituted serine, other carbohydrate modified amino acids, keto-containing amino acids, amino acids comprising polyethylene glycol or polyether, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, amino acids with an elongated side chains as compared to natural amino acids, e.g., polyethers or long chain hydrocarbons, e.g., greater than about 5 or greater than about 10 carbons, carbon-linked sugar-containing amino acids, redox-active amino acids, amino thioacid containing amino acids, and amino acids comprising one or more toxic moiety.

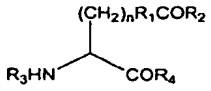
[98] Exemplary non-naturally encoded amino acids useful for reactions with water soluble polymers include those with carbonyl, aminooxy, hydroxylamine, hydrazide, semicarbazide, azide and alkyne reactive groups. In some embodiments, non-naturally encoded amino acids comprise a saccharide moiety. Examples of such amino acids include *N*-acetyl-L-glucosaminyl-L-serine, *N*-acetyl-L-galactosaminyl-L-serine, *N*-acetyl-L-glucosaminyl-L-threonine, *N*-acetyl-L-glucosaminyl-L-asparagine and *O*-mannosaminyl-L-serine. Examples of such amino acids also include examples where the naturally-occuring N- or O- linkage between the amino acid and the saccharide is replaced by a covalent linkage not commonly found in nature – e.g., an alkene, an oxime, a thioether, an amide and the like. Examples of such amino acids also include saccharides that are not commonly found in naturally-occuring proteins such as 2-deoxy-glucose, 2-deoxygalactose and the like.

[99] Many of the non-naturally encoded amino acids provided herein are commercially available, e.g., from Sigma-Aldrich (St. Louis, MO, USA), Novabiochem (a division of EMD Biosciences, Darmstadt, Germany), or Peptech (Burlington, MA, USA). Those that are not commercially available are optionally synthesized as provided herein or using standard methods known to those of skill in the art. For organic synthesis techniques, see, e.g., Organic Chemistry by Fessendon and Fessendon, (1982, Second Edition, Willard Grant Press, Boston Mass.); Advanced Organic Chemistry by March (Third Edition, 1985, Wiley and Sons, New York); and Advanced Organic Chemistry by Carey and Sundberg (Third Edition, Parts A and B, 1990, Plenum Press, New York). *See, also*, U.S. Patent Application Publications 2003/0082575 and 2003/0108885, incorporated by reference.

A. Carbonyl reactive groups

[100] Amino acids with a carbonyl reactive group allow for a variety of reactions to link molecules (e.g., PEG or other water soluble molecules) via nucleophilic addition or aldol condensation reactions among others.

[101] Exemplary carbonyl-containing amino acids can be represented as follows:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, or substituted aryl; R_2 is H, alkyl, aryl, substituted alkyl, and substituted aryl; and R_3 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_4 is H, an amino acid, a polypeptide, or a carboxy terminus modification group. In some embodiments, n is 1, R_1 is phenyl and R_2 is a simple alkyl (i.e., methyl, ethyl, or propyl) and the ketone moiety is positioned in the *para* position relative to the alkyl side chain. In some embodiments, n is 1, R_1 is phenyl and R_2 is a simple alkyl (i.e., methyl, ethyl, or propyl) and the ketone moiety is positioned in the *meta* position relative to the alkyl side chain.

[102] The synthesis of *p*-acetyl-(+/-)-phenylalanine and *m*-acetyl-(+/-)-phenylalanine is described in Zhang, Z., et al., Biochemistry 42: 6735-6746 (2003), incorporated by reference. Other carbonyl-containing amino acids can be similarly prepared by one skilled in the art.

[103] In some embodiments, a polypeptide comprising a non-naturally encoded amino acid is chemically modified to generate a reactive carbonyl functional group. For instance, an aldehyde functionality useful for conjugation reactions can be generated from a functionality having adjacent amino and hydroxyl groups. Where the biologically active molecule is a polypeptide, for example, an *N*-terminal serine or threonine (which may be normally present or may be exposed via chemical or enzymatic digestion) can be used to generate an aldehyde functionality under mild oxidative cleavage conditions using periodate. *See, e.g.*, Gaertner, *et. al., Bioconjug. Chem.* 3: 262-268 (1992); Geoghegan, K. & Stroh, J., *Bioconjug. Chem.* 3:138-146 (1992); Gaertner *et al., J. Biol. Chem.* 269:7224-7230 (1994). However, methods known in the art are restricted to the amino acid at the *N*-terminus of the peptide or protein.

[104] In the present invention, a non-naturally encoded amino acid bearing adjacent hydroxyl and amino groups can be incorporated into the polypeptide as a "masked" aldehyde functionality. For example, 5-hydroxylysine bears a hydroxyl group adjacent to the epsilon amine. Reaction conditions for generating the aldehyde typically involve addition of molar excess of sodium metaperiodate under mild conditions to avoid oxidation at other sites within the polypeptide. The pH of the oxidation reaction is typically about 7.0. A typical

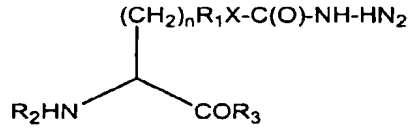
reaction involves the addition of about 1.5 molar excess of sodium meta periodate to a buffered solution of the polypeptide, followed by incubation for about 10 minutes in the dark. *See, e.g.* U.S. Patent No. 6,423,685.

[105] The carbonyl functionality can be reacted selectively with a hydrazine-hydrazide-, hydroxylamine-, or semicarbazide-containing reagent under mild conditions in aqueous solution to form the corresponding hydrazone, oxime, or semicarbazone linkages, respectively, that are stable under physiological conditions. *See, e.g.*, Jencks, W. P., *J. Am. Chem. Soc.* 81, 475-481 (1959); Shao, J. and Tam, J. P., *J. Am. Chem. Soc.* 117:3893-3899 (1995). Moreover, the unique reactivity of the carbonyl group allows for selective modification in the presence of the other amino acid side chains. *See, e.g.*, Cornish, V. W., *et al.*, *J. Am. Chem. Soc.* 118:8150-8151 (1996); Geoghegan, K. F. & Stroh, J. G., *Bioconjug. Chem.* 3:138-146 (1992); Mahal, L. K., *et al.*, *Science* 276:1125-1128 (1997).

B. Hydrazine, hydrazide or semicarbazide reactive groups

[106] Non-naturally encoded amino acids containing a nucleophilic group, such as a hydrazine, hydrazide or semicarbazide, allow for reaction with a variety of electrophilic groups to form conjugates (e.g., with PEG or other water soluble polymers).

[107] Exemplary hydrazine, hydrazide or semicarbazide -containing amino acids can be represented as follows:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, or substituted aryl or not present; X, is O, N, or S or not present; R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

[108] In some embodiments, n is 4, R_1 is not present, and X is N. In some embodiments, n is 2, R_1 is not present, and X is not present. In some embodiments, n is 1, R_1 is phenyl, X is O, and the oxygen atom is positioned *para* to the alphatic group on the aryl ring.

[109] Hydrazide-, hydrazine-, and semicarbazide-containing amino acids are available from commercial sources. For instance, L-glutamate-γ-hydrazide is available from

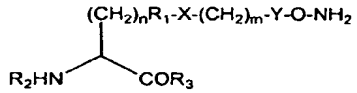
Sigma Chemical (St. Louis, MO). Other amino acids not available commercially can be prepared by one skilled in the art. *See, e.g.,* U.S. Pat. No. 6,281,211.

[110] Polypeptides containing non-naturally encoded amino acids that bear hydrazide, hydrazine or semicarbazide functionalities can be reacted efficiently and selectively with a variety of molecules that contain aldehydes or other functional groups with similar chemical reactivity. *See, e.g.,* Shao, J. and Tam, J., *J. Am. Chem. Soc.* 117:3893-3899 (1995). The unique reactivity of hydrazide, hydrazine and semicarbazide functional groups makes them significantly more reactive toward aldehydes, ketones and other electrophilic groups as compared to the nucleophilic groups present on the 20 common amino acids (e.g., the hydroxyl group of serine or threonine or the amino groups of lysine and the N-terminus).

C. Aminooxy-containing amino acids

[111] Non-naturally encoded amino acids containing an aminooxy (also called a hydroxylamine) group allow for reaction with a variety of electrophilic groups to form conjugates (e.g., with PEG or other water soluble polymers). Like hydrazines, hydrazides and semicarbazides, the enhanced nucleophilicity of the aminooxy group permits it to react efficiently and selectively with a variety of molecules that contain aldehydes or other functional groups with similar chemical reactivity. *See, e.g.,* Shao, J. and Tam, J., *J. Am. Chem. Soc.* 117:3893-3899 (1995); H. Hang and C. Bertozzi, *Acc. Chem. Res.* 34: 727-736 (2001). Whereas the result of reaction with a hydrazine group is the corresponding hydrazone, however, an oxime results generally from the reaction of an aminooxy group with a carbonyl-containing group such as a ketone.

[112] Exemplary amino acids containing aminooxy groups can be represented as follows:



wherein n is 0-10; R1 is an alkyl, aryl, substituted alkyl, or substituted aryl or not present; X is O, N, S or not present; m is 0-10; Y = C(O) or not present; R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group. In some embodiments, n is 1, R_1 is phenyl, X is O, m is 1, and Y is present. In some embodiments, n is 2, R_1 and X are not present, m is 0, and Y is not present.

[113] Aminooxy-containing amino acids can be prepared from readily available amino acid precursors (homoserine, serine and threonine). *See, e.g.*, M. Carrasco and R. Brown, *J. Org. Chem.* 68: 8853-8858 (2003). Certain aminooxy-containing amino acids, such as L-2-amino-4-(aminooxy)butyric acid), have been isolated from natural sources (Rosenthal, G. et al., Life Sci. 60: 1635-1641 (1997). Other aminooxy-containing amino acids can be prepared by one skilled in the art.

D. Azide and alkyne reactive groups

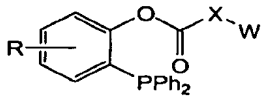
[114] The unique reactivity of azide and alkyne functional groups makes them extremely useful for the selective modification of polypeptides and other biological molecules. Organic azides, particularly alphatic azides, and alkynes are generally stable toward common reactive chemical conditions. In particular, both the azide and the alkyne functional groups are inert toward the side chains (i.e., R groups) of the 20 common amino acids found in naturally-occuring polypeptides. When brought into close proximity, however, the "spring-loaded" nature of the azide and alkyne groups is revealed and they react selectively and efficiently via Huisgen [3+2] cycloaddition reaction to generate the corresponding triazole. *See, e.g.*, Chin J., *et al.*, *Science* 301:964-7 (2003); Wang, Q., *et al.*, *J. Am. Chem. Soc.* 125, 3192-3193 (2003); Chin, J. W., *et al.*, *J. Am. Chem. Soc.* 124:9026-9027 (2002).

[115] Because the Huisgen cycloaddition reaction involves a selective cycloaddition reaction (*see, e.g.*, Padwa, A., in COMPREHENSIVE ORGANIC SYNTHESIS, Vol. 4, (ed. Trost, B. M., 1991), p. 1069-1109; Huisgen, R. in 1,3-DIPOLAR CYCLOADDITION CHEMISTRY, (ed. Padwa, A., 1984) , p. 1-176) rather than a nucleophilic substitution, the incorporation of non-naturally encoded amino acids bearing azide and alkyne-containing side chains permits the resultant polypeptides to be modified selectively at the position of the non-naturally encoded amino acid. Cycloaddition reaction involving azide or alkyne-containing hGH can be carried out at room temperature under aqueous conditions by the addition of Cu(II) (e.g., in the form of a catalytic amount of $CuSO_4$) in the presence of a reducing agent for reducing Cu(II) to Cu(I), in situ, in catalytic amount. *See, e.g.*, Wang, Q., *et al.*, *J. Am. Chem. Soc.* 125, 3192-3193 (2003); Tornoe, C. W., *et al.*, *J. Org. Chem.* 67:3057-3064 (2002); Rostovtsev, *et al.*, *Angew. Chem. Int. Ed.* 41:2596-2599 (2002). Exemplary reducing agents include, e.g., ascorbate, metallic copper, quinine, hydroquinone, vitamin K, glutathione, cysteine, Fe^{2+}, Co^{2+}, and an applied electric potential.

[116] In some cases, where a Huisgen [3+2] cycloaddition reaction between an azide and an alkyne is desired, the hGH polypeptide comprises a non-naturally encoded amino acid comprising an alkyne moiety and the water soluble polymer to be attached to the amino acid comprises an azide moiety. Alternatively, the converse reaction (i.e., with the azide moiety on the amino acid and the alkyne moiety present on the water soluble polymer) can also be performed.

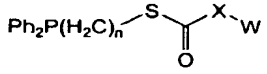
[117] The azide functional group can also be reacted selectively with a water soluble polymer containing an aryl ester and appropriately functionalized with an aryl phosphine moiety to generate an amide linkage. The aryl phosphine group reduces the azide in situ and the resulting amine then reacts efficiently with a proximal ester linkage to generate the corresponding amide. *See, e.g.*, E. Saxon and C. Bertozzi, *Science* 287, 2007-2010 (2000). The azide-containing amino acid can be either an alkyl azide (e.g., 2-amino-6-azido-1-hexanoic acid) or an aryl azide (p-azido-phenylalanine).

[118] Exemplary water soluble polymers containing an aryl ester and a phosphine moiety can be represented as follows:



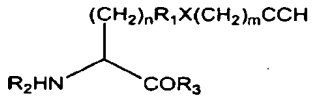
wherein X can be O, N, S or not present, Ph is phenyl, W is a water soluble polymer and R can be H, alkyl, aryl, substituted alkyl and substituted aryl groups. Exemplary R groups include but are not limited to $-CH_2$, $-C(CH_3)_3$, -OR', -NR'R", -SR', -halogen, -C(O)R', -CONR'R", $-S(O)_2R'$, $-S(O)_2NR'R"$, -CN and $-NO_2$. R', R", R'" and R'"" each independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, *e.g.*, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R'"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, $-CF_3$ and $-CH_2CF_3$) and acyl (*e.g.*, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like).

[119] The azide functional group can also be reacted selectively with a water soluble polymer containing a thioester and appropriately functionalized with an aryl phosphine moiety to generate an amide linkage. The aryl phosphine group reduces the azide in situ and the resulting amine then reacts efficiently with the thioester linkage to generate the corresponding amide. Exemplary water soluble polymers containing a thioester and a phosphine moiety can be represented as follows:



wherein n is 1-10; X can be O, N, S or not present, Ph is phenyl, and W is a water soluble polymer.

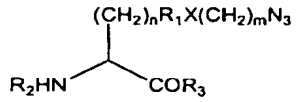
[120] Exemplary alkyne-containing amino acids can be represented as follows:



wherein n is 0-10; R1 is an alkyl, aryl, substituted alkyl, or substituted aryl or not present; X is O, N, S or not present; m is 0-10, R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group. In some embodiments, n is 1, R_1 is phenyl, X is not present, m is 0 and the acetylene moiety is positioned in the para position relative to the alkyl side chain. In some embodiments, n is 1, R_1 is phenyl, X is O, m is 1 and the propargyloxy group is positioned in the *para* position relative to the alkyl side chain (i.e., O-propargyl-tyrosine). In some embodiments, n is 1, R_1 and X are not present and m is 0 (i.e., proparylglycine).

[121] Alkyne-containing amino acids are commercially available. For example, propargylglycine is commercially available from Peptech (Burlington, MA). Alternatively, alkyne-containing amino acids can be prepared according to standard methods. For instance, *p*-propargyloxyphenylalanine can be synthesized, e.g., as described in Deiters, A., *et al., J. Am. Chem. Soc.* 125: 11782-11783 (2003), and 4-alkynyl-L-phenylalanine can be synthesized as described in Kayser, B., *et al., Tetrahedron* 53(7): 2475-2484 (1997). Other alkyne-containing amino acids can be prepared by one skilled in the art.

[122] Exemplary azide-containing amino acids can be represented as follows:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, substituted aryl or not present; X is O, N, S or not present; m is 0-10; R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group. In some embodiments, n is 1, R_1 is phenyl, X is not present, m is 0 and the azide moiety is positioned *para* to the alkyl side chain. In some embodiments, n is 0-4 and R_1 and X are not present, and m=0. In some embodiments, n is 1, R_1 is phenyl, X is O, m is 2 and the β-azidoethoxy moiety is positioned in the *para* position relative to the alkyl side chain.

[123] Azide-containing amino acids are available from commercial sources. For instance, 4-azidophenylalanine can be obtained from Chem-Impex International, Inc. (Wood Dale, IL). For those azide-containing amino acids that are not commercially available, the azide group can be prepared relatively readily using standard methods known to those of skill in the art, e.g., via displacement of a suitable leaving group (e.g., halide, mesylate, tosylate) or via opening of a suitably protected lactone. *See, e.g.,* Advanced Organic Chemistry by March (Third Edition, 1985, Wiley and Sons, New York).

E. Aminothiol reactive groups

[124] The unique reactivity of beta-substituted aminothiol functional groups makes them extremely useful for the selective modification of polypeptides and other biological molecules that contain aldehyde groups via formation of the thiazolidine. *See, e.g.,* J. Shao and J. Tam, *J. Am. Chem. Soc.* 1995, 117 (14) 3893-3899. In some embodiments, beta-substituted aminothiol amino acids can be incorporated into hGH polypeptides and then reacted with water soluble polymers comprising an aldehyde functionality. In some embodiments, a water soluble polymer, drug conjugate or other payload can be coupled to an hGH polypeptide comprising a beta-substituted aminothiol amino acid via formation of the thiazolidine.

V. In vivo generation of hGH comprising non-genetically-encoded amino acids

[125] hGH polypeptides of the invention can be generated *in vivo* using modified tRNA and tRNA synthetases to add to or substitute amino acids that are not encoded in naturally-occurring systems.

[126] Methods for generating tRNAs and tRNA synthetases which use amino acids that are not encoded in naturally-occurring systems are described in, e.g., U.S. Patent Application Publications 2003/0082575 and 2003/0108885. These methods involve generating a translational machinery that functions independently of the synthetases and tRNAs endogenous to the translation system (and are therefore sometimes referred to as "orthogonal"). Typically, the translation system comprises an orthogonal tRNA (O-tRNA) and an orthogonal aminoacyl tRNA synthetase (O-RS). Typically, the O-RS preferentially aminoacylates the O-tRNA with at least one non-naturally occurring amino acid in the translation system and the O-tRNA recognizes at least one selector codon that is not recognized by other tRNAs in the system. The translation system thus inserts the non-naturally-encoded amino acid into a protein produced in the system, in response to an encoded selector codon, thereby "substituting" an amino acid into a position in the encoded polypeptide.

[127] A number of orthogonal tRNAs and aminoacyl tRNA synthetases have been described in the art for inserting particular synthetic amino acids into polypeptides. For example, keto-specific O-tRNA/aminoacyl-tRNA synthetases are described in Wang, L., *et al.*, *Proc. Natl. Acad. Sci. USA* 100:56-61 (2003) and Zhang, Z. et al., *Biochem.* 42(22):6735-6746 (2003). Exemplary O-RS, or portions thereof, are encoded by polynucleotide sequences and include amino acid sequences disclosed in U.S. Patent Application Publications 2003/0082575 and 2003/0108885, each incorporated herein by reference. Corresponding O-tRNA molecules for use with the O-RSs are also described in U.S. Patent Application Publications 2003/0082575 and 2003/0108885.

[128] An example of an azide-specific O-tRNA/aminoacyl-tRNA synthetase system is described in Chin, J. W., *et al.*, *J. Am. Chem. Soc.* 124:9026-9027 (2002). Exemplary O-RS and O-tRNA sequences for inserting *p*-azido-L-Phe include, e.g., nucleotide sequences SEQ ID NOs: 14-16 and 29-32 and amino acid sequences SEQ ID NOs: 46-48 and 61-64 as disclosed in U.S. Patent Application Publication 2003/0108885. Other examples of O-tRNA/aminoacyl-tRNA synthetase pairs specific to particular non-naturally encoded amino acids are described in U.S. Patent Application Publication 2003/0082575.

[129] O-RS and O-tRNA that incorporate both keto- and azide-containing amino acids in *S. cerevisiae* are described in Chin, J. W., *et al.*, *Science* 301:964-967 (2003).

[130] Use of O-tRNA/aminoacyl-tRNA synthetases involves selection of a specific codon which encodes the non-naturally encoded amino acid. While any codon can be used, it is generally desirable to select a codon that is rarely or never used in the cell in

which the O-tRNA/aminoacyl-tRNA synthetase is expressed. For example, exemplary codons include nonsense codon such as stop codons (amber, ochre, and opal), four or more base codons and other natural three-base codons that are rarely or unused.

[131] Specific selector codon(s) can be introduced into appropriate positions in the hGH polynucleotide coding sequence using mutagenesis methods known in the art (e.g., site-specific mutagenesis, cassette mutagenesis, restriction selection mutagenesis, etc.).

[132] Methods for generating components of the protein biosynthetic machinery, such as O-RSs, O-tRNAs, and orthogonal O-tRNA/O-RS pairs that can be used to incorporate a non-naturally encoded amino acid are described in Wang, L., *et al., Science* 292: 498-500 (2001); Chin, J. W., *et al., J. Am. Chem. Soc.* 124:9026-9027 (2002); Zhang, Z. *et al., Biochemistry* 42: 6735-6746 (2003). Methods and compositions for the in vivo incorporation of non-naturally encoded amino acids are described in U.S. Patent Application Publication 2003/0082575. Methods for selecting an orthogonal tRNA-tRNA synthetase pair for use in *in vivo* translation system of an organism are also described in U.S. Patent Application Publications 2003/0082575 and 2003/0108885.

[133] Methods for producing at least one recombinant orthogonal aminoacyl-tRNA synthetase (O-RS) comprise: (a) generating a library of (optionally mutant) RSs derived from at least one aminoacyl-tRNA synthetase (RS) from a first organism, e.g., a prokaryotic organism, such as *Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Halobacterium, Escherichia coli, A. fulgidus, P. furiosus, P. horikoshii, A. pernix, T. thermophilus,* or the like, or a eukaryotic organism; (b) selecting (and/or screening) the library of RSs (optionally mutant RSs) for members that aminoacylate an orthogonal tRNA (O-tRNA) in the presence of a non-naturally encoded amino acid and a natural amino acid, thereby providing a pool of active (optionally mutant) RSs; and/or, (c) selecting (optionally through negative selection) the pool for active RSs (e.g., mutant RSs) that preferentially aminoacylate the O-tRNA in the absence of the non-naturally encoded amino acid, thereby providing the at least one recombinant O-RS; wherein the at least one recombinant O-RS preferentially aminoacylates the O-tRNA with the non-naturally encoded amino acid.

[134] In one embodiment, the RS is an inactive RS. The inactive RS can be generated by mutating an active RS. For example, the inactive RS can be generated by mutating at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, or at least about 10 or more amino acids to different amino acids, e.g., alanine.

[135] Libraries of mutant RSs can be generated using various mutagenesis techniques known in the art. For example, the mutant RSs can be generated by site-specific mutations, random mutations, diversity generating recombination mutations, chimeric constructs, and by other methods described herein or known in the art.

[136] In one embodiment, selecting (and/or screening) the library of RSs (optionally mutant RSs) for members that are active, e.g., that aminoacylate an orthogonal tRNA (O-tRNA) in the presence of a non-naturally encoded amino acid and a natural amino acid, includes: introducing a positive selection or screening marker, e.g., an antibiotic resistance gene, or the like, and the library of (optionally mutant) RSs into a plurality of cells, wherein the positive selection and/or screening marker comprises at least one selector codon, e.g., an amber, ochre, or opal codon; growing the plurality of cells in the presence of a selection agent; identifying cells that survive (or show a specific response) in the presence of the selection and/or screening agent by suppressing the at least one selector codon in the positive selection or screening marker, thereby providing a subset of positively selected cells that contains the pool of active (optionally mutant) RSs. Optionally, the selection and/or screening agent concentration can be varied.

[137] In one aspect, the positive selection marker is a chloramphenicol acetyltransferase (CAT) gene and the selector codon is an amber stop codon in the CAT gene. Optionally, the positive selection marker is a β-lactamase gene and the selector codon is an amber stop codon in the β-lactamase gene. In another aspect the positive screening marker comprises a fluorescent or luminescent screening marker or an affinity based screening marker (e.g., a cell surface marker).

[138] In one embodiment, negatively selecting or screening the pool for active RSs (optionally mutants) that preferentially aminoacylate the O-tRNA in the absence of the non-naturally encoded amino acid includes: introducing a negative selection or screening marker with the pool of active (optionally mutant) RSs from the positive selection or screening into a plurality of cells of a second organism, wherein the negative selection or screening marker comprises at least one selector codon (e.g., an antibiotic resistance gene, e.g., a chloramphenicol acetyltransferase (CAT) gene); and, identifying cells that survive or show a specific screening response in a first medium supplemented with the non-naturally encoded amino acid and a screening or selection agent, but fail to survive or to show the specific response in a second medium not supplemented with the non-naturally encoded amino acid and the selection or screening agent, thereby providing surviving cells or screened

cells with the at least one recombinant O-RS. For example, a CAT identification protocol optionally acts as a positive selection and/or a negative screening in determination of appropriate O-RS recombinants. For instance, a pool of clones is optionally replicated on growth plates containing CAT (which comprises at least one selctor codon) either with or without one or more non-naturally encoded amino acid. Colonies growing exclusively on the plates containing non-naturally encoded amino acids are thus regarded as containing recombinant O-RS. In one aspect, the concentration of the selection (and/or screening) agent is varied. In some aspects the first and second organisms are different. Thus, the first and/or second organism optionally comprises: a prokaryote, a eukaryote, a mammal, an *Escherichia coli*, a fungi, a yeast, an archaebacterium, a eubacterium, a plant, an insect, a protist, etc. In other embodiments, the screening marker comprises a fluorescent or luminescent screening marker or an affinity based screening marker.

[139] In another embodiment, screening or selecting (e.g., negatively selecting) the pool for active (optionally mutant) RSs includes: isolating the pool of active mutant RSs from the positive selection step (b); introducing a negative selection or screening marker, wherein the negative selection or screening marker comprises at least one selector codon (e.g., a toxic marker gene, e.g., a ribonuclease barnase gene, comprising at least one selector codon), and the pool of active (optionally mutant) RSs into a plurality of cells of a second organism; and identifying cells that survive or show a specific screening response in a first medium not supplemented with the non-naturally encoded amino acid, but fail to survive or show a specific screening response in a second medium supplemented with thc non-naturally encoded amino acid, thereby providing surviving or screened cells with the at least one recombinant O-RS, wherein the at least one recombinant O-RS is specific for the non-naturally encoded amino acid. In one aspect, the at least one selector codon comprises about two or more selector codons. Such embodiments optionally can include wherein the at least one selector codon comprises two or more selector codons, and wherein the first and second organism are different (e.g., each organism is optionally, e.g., a prokaryote, a eukaryote, a mammal, an *Escherichia coli*, a fungi, a yeast, an archaebacteria, a eubacteria, a plant, an insect, a protist, etc.). Also, some aspects include wherein the negative selection marker comprises a ribonuclease barnase gene (which comprises at least one selector codon). Other aspects include wherein the screening marker optionally comprises a fluorescent or luminescent screening marker or an affinity based screening marker. In the embodiments herein, the screenings and/or selections optionally include variation of the screening and/or selection stringency.

[140] In one embodiment, the methods for producing at least one recombinant orthogonal aminoacyl-tRNA synthetase (O-RS) can further comprise:(d) isolating the at least one recombinant O-RS; (e) generating a second set of O-RS (optionally mutated) derived from the at least one recombinant O-RS; and, (f) repeating steps (b) and (c) until a mutated O-RS is obtained that comprises an ability to preferentially aminoacylate the O-tRNA. Optionally, steps (d)-(f) are repeated, e.g., at least about two times. In one aspect, the second set of mutated O-RS derived from at least one recombinant O-RS can be generated by mutagenesis, e.g., random mutagenesis, site-specific mutagenesis, recombination or a combination thereof.

[141] The stringency of the selection/screening steps, e.g., the positive selection/screening step (b), the negative selection/screening step (c) or both the positive and negative selection/screening steps (b) and (c), in the above-described methods, optionally includes varying the selection/screening stringency. In another embodiment, the positive selection/screening step (b), the negative selection/screening step (c) or both the positive and negative selection/screening steps (b) and (c) comprise using a reporter, wherein the reporter is detected by fluorescence-activated cell sorting (FACS) or wherein the reporter is detected by luminescence. Optionally, the reporter is displayed on a cell surface, on a phage display or the like and selected based upon affinity or catalytic activity involving the non-naturally encoded amino acid or an analogue. In one embodiment, the mutated synthetase is displayed on a cell surface, on a phage display or the like.

[142] Methods for producing a recombinant orthogonal tRNA (O-tRNA) include: (a) generating a library of mutant tRNAs derived from at least one tRNA, e.g., a suppressor tRNA, from a first organism; (b) selecting (e.g., negatively selecting) or screening the library for (optionally mutant) tRNAs that are aminoacylated by an aminoacyl-tRNA synthetase (RS) from a second organism in the absence of a RS from the first organism, thereby providing a pool of tRNAs (optionally mutant); and, (c) selecting or screening the pool of tRNAs (optionally mutant) for members that are aminoacylated by an introduced orthogonal RS (O-RS), thereby providing at least one recombinant O-tRNA; wherein the at least one recombinant O-tRNA recognizes a selector codon and is not efficiency recognized by the RS from the second organism and is preferentially aminoacylated by the O-RS. In some embodiments the at least one tRNA is a suppressor tRNA and/or comprises a unique three base codon of natural and/or unnatural bases, or is a nonsense codon, a rare codon, an unnatural codon, a codon comprising at least 4 bases, an amber codon, an ochre codon, or an opal stop codon. In one embodiment, the recombinant O-tRNA possesses an improvement of

orthogonality. It will be appreciated that in some embodiments, O-tRNA is optionally imported into a first organism from a second organism without the need for modification. In various embodiments, the first and second organisms are either the same or different and are optionally chosen from, e.g., prokaryotes (e.g., *Methanococcus jannaschii*, *Methanobacteium thermoautotrophicum*, *Escherichia coli*, *Halobacterium*, etc.), eukaryotes, mammals, fungi, yeasts, archaebacteria, eubacteria, plants, insects, protists, etc. Additionally, the recombinant tRNA is optionally aminoacylated by a non-naturally encoded amino acid, wherein the non-naturally encoded amino acid is biosynthesized in vivo either naturally or through genetic manipulation. The non-naturally encoded amino acid is optionally added to a growth medium for at least the first or second organism.

[143] In one aspect, selecting (e.g., negatively selecting) or screening the library for (optionally mutant) tRNAs that are aminoacylated by an aminoacyl-tRNA synthetase (step (b)) includes: introducing a toxic marker gene, wherein the toxic marker gene comprises at least one of the selector codons (or a gene that leads to the production of a toxic or static agent or a gene essential to the organism wherein such marker gene comprises at least one selector codon) and the library of (optionally mutant) tRNAs into a plurality of cells from the second organism; and, selecting surviving cells, wherein the surviving cells contain the pool of (optionally mutant) tRNAs comprising at least one orthogonal tRNA or nonfunctional tRNA. For example, surviving cells can be selected by using a comparison ratio cell density assay.

[144] In another aspect, the toxic marker gene can include two or more selector codons. In another embodiment of the methods, the toxic marker gene is a ribonuclease barnase gene, where the ribonuclease barnase gene comprises at least one amber codon. Optionally, the ribonuclease barnase gene can include two or more amber codons.

[145] In one embodiment, selecting or screening the pool of (optionally mutant) tRNAs for members that are aminoacylated by an introduced orthogonal RS (O-RS) can include: introducing a positive selection or screening marker gene, wherein the positive marker gene comprises a drug resistance gene (e.g., β-lactamase gene, comprising at least one of the selector codons, such as at least one amber stop codon) or a gene essential to the organism, or a gene that leads to detoxification of a toxic agent, along with the O-RS, and the pool of (optionally mutant) tRNAs into a plurality of cells from the second organism; and, identifying surviving or screened cells grown in the presence of a selection or screening agent, e.g., an antibiotic, thereby providing a pool of cells possessing the at least one

recombinant tRNA, where the at least recombinant tRNA is aminoacylated by the O-RS and inserts an amino acid into a translation product encoded by the positive marker gene, in response to the at least one selector codons. In another embodiment, the concentration of the selection and/or screening agent is varied.

[146] Methods for generating specific O-tRNA/O-RS pairs are provided. Methods include: (a) generating a library of mutant tRNAs derived from at least one tRNA from a first organism; (b) negatively selecting or screening the library for (optionally mutan) tRNAs that are aminoacylated by an aminoacyl-tRNA synthetase (RS) from a second organism in the absence of a RS from the first organism, thereby providing a pool of (optionally mutant) tRNAs; (c) selecting or screening the pool of (optionally mutant) tRNAs for members that are aminoacylated by an introduced orthogonal RS (O-RS), thereby providing at least one recombinant O-tRNA. The at least one recombinant O-tRNA recognizes a selector codon and is not efficiency recognized by the RS from the second organism and is preferentially aminoacylated by the O-RS. The method also includes (d) generating a library of (optionally mutant) RSs derived from at least one aminoacyl-tRNA synthetase (RS) from a third organism; (e) selecting or screening the library of mutant RSs for members that preferentially aminoacylate the at least one recombinant O-tRNA in the presence of a non-naturally encoded amino acid and a natural amino acid, thereby providing a pool of active (optionally mutant) RSs; and, (f) negatively selecting or screening the pool for active (optionally mutant) RSs that preferentially aminoacylate the at least one recombinant O-tRNA in the absence of the non-naturally encoded amino acid, thereby providing the at least one specific O-tRNA/O-RS pair, wherein the at least one specific O-tRNA/O-RS pair comprises at least one recombinant O-RS that is specific for the non-naturally encoded amino acid and the at least one recombinant O-tRNA. Specific O-tRNA/O-RS pairs produced by the methods are included. For example, the specific O-tRNA/O-RS pair can include, e.g., a mutRNATyr-mutTyrRS pair, such as a mutRNATyr-SS12TyrRS pair, a mutRNALeu-mutLeuRS pair, a mutRNAThr-mutThrRS pair, a mutRNAGlu-mutGluRS pair, or the like. Additionally, such methods include wherein the first and third organism are the same (e.g., *Methanococcus jannaschii*).

[147] Methods for selecting an orthogonal tRNA-tRNA synthetase pair for use in an *in vivo* translation system of a second organism are also included in the present invention. The methods include: introducing a marker gene, a tRNA and an aminoacyl-tRNA synthetase (RS) isolated or derived from a first organism into a first set of cells from the second organism; introducing the marker gene and the tRNA into a duplicate cell set from a

second organism; and, selecting for surviving cells in the first set that fail to survive in the duplicate cell set or screening for cells showing a specific screening response that fail to give such response in the duplicate cell set, wherein the first set and the duplicate cell set are grown in the presence of a selection or screening agent, wherein the surviving or screened cells comprise the orthogonal tRNA-tRNA synthetase pair for use in the in the in vivo translation system of the second organism. In one embodiment, comparing and selecting or screening includes an in vivo complementation assay. The concentration of the selection or screening agent can be varied.

[148] The organisms of the present invention comprise a variety of organism and a variety of combinations. For example, the first and the second organisms of the methods of the present invention can be the same or different. In one embodiment, the organisms are optionally a prokaryotic organism, e.g., *Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Halobacterium, Escherichia coli, A. fulgidus, P. furiosus, P. horikoshii, A. pernix, T. thermophilus,* or the like. Alternatively, the organisms optionally comprise a eukaryotic organism, e.g., plants (e.g., complex plants such as monocots, or dicots), algae, protists, fungi (e.g., yeast, etc), animals (e.g., mammals, insects, arthropods, etc.), or the like. In another embodiment, the second organism is a prokaryotic organism, e.g., *Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Halobacterium, Escherichia coli, A. fulgidus, Halobacterium, P. furiosus, P. horikoshii, A. pernix, T. thermophilus,* or the like. Alternatively, the second organism can be a eukaryotic organism, e.g., a yeast, a animal cell, a plant cell, a fungus, a mammalian cell, or the like. In various embodiments the first and second organisms are different.

VI. Location of non-naturally-occurring amino acids in hGH

[149] The present invention contemplates insertion of one or more non-naturally-occurring amino acids into hGH. One or more non-naturally-occurring amino acids will generally be inserted at a particular position which does not disrupt activity of the polypeptide. This can be achieved by making "conservative" substitutions, e.g., substituting hydrophobic amino acids with hydrophobic amino acids, bulky amino acids for bulky amino acids, hydrophilic amino acids for hydrophilic amino acids) and/or inserting the non-naturally-occurring amino acid in a location that is not required for activity.

[150] Regions of hGH can be illustrated as follows, wherein the amino acid positions in hGH are indicated in the middle row:

	Helix A		Helix B		Helix C		Helix D	
[1-5] - N-term	[6-33] -	[34-74] - A-B loop	[75-96] -	[97-105] - B-C loop	[106-129] -	[130-153] - C-D loop	[154-183] -	[184-191] C-term

[151] A variety of biochemical and structural approaches can be employed to determine the optimum sites for substitution with a non-naturally encoded amino acid within the hGH polypeptide. For example, locations in the polypeptide that are required for biological activity of hGH can be identified using alanine scanning or homolog scanning methods known in the art. *See, e.g.*, Cunningham, B. and Wells, J., *Science*, 244:1081-1085 (1989) (identifying 14 residues that are critical for hGH bioactivity) and Cunningham, B., *et al. Science* 243: 1330-1336 (1989) (identifying antibody and receptor epitopes using homolog scanning mutagenesis). In general, residues other than those identified as critical to biological activity by alanine or homolog scanning mutagenesis are good candidates for substitution with a non-naturally encoded amino acid.

[152] The structure and activity of naturally-occurring mutants of hGH that contain deletions can also be examined to determine regions of the protein that are likely to be tolerant of substitution with a non-naturally encoded amino acid. *See, e.g.*, Kostyo *et al., Biochem. Biophys. Acta*, 925: 314 (1987); Lewis, U., *et al., J. Biol. Chem.*, 253:2679-2687 (1978). In a similar manner, protease digestion and monoclonal antibodies can be used to identify regions of hGH that are responsible for binding the hGH receptor. See, e.g., Cunningham, B., *et al. Science* 243: 1330-1336 (1989); Mills, J., *et al., Endocrinology*, 107:391-399 (1980); Li, C., *Mol. Cell. Biochem.*, 46:31-41 (1982) (indicating that amino acids between residues 134-149 can be deleted without a loss of activity). Once residues that are likely to be intolerant to substitution with non-naturally encoded amino acids have been eliminated, the impact of proposed substitutions at each of the remaining positions can be examined from the three-dimensional crystal structure of hGH and its binding protein. *See* de Vos, A., *et al.*, *Science*, 255:306-312 (1992); all crystal structures of hGH are available in the Protein Data Bank (PDB, www.rcsb.org), a centralized database containing three-dimensional structural data of large molecules of proteins and nucleic acids. Thus, those of skill in the art can readily identify amino acid positions that can be substituted with non-naturally encoded amino acids.

[153] In some embodiments, hGH polypeptides of the invention comprise at least one non-naturally-occurring amino acid substituted for at least one amino acid located in at least one region of hGH selected from the group consisting of the N-terminus (1-5), the N-terminal end of the A-B loop (32-46); the B-C loop (97-105), the C-D loop (132-149), and

the C-terminus (184-191). In some embodiments, the non-naturally encoded amino acid is substituted at one or more of the following positions of hGH: before position 1 (i.e., at the N-terminus), 1, 2, 3, 4, 5, 9, 11, 12, 15, 16, 19, 22, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,43, 44, 45, 46, 47, 48, 49, 52, 55, 57, 59, 65, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 109, 111, 112, 113, 115, 116, 119, 120, 122, 123, 126, 127, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 158, 159, 161, 168, 172, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192 (i.e., at the carboxyl terminus of the protein) or combinations thereof.

[154] Exemplary sites of incorporation of a non-naturally encoded amino acid include 29, 30, 33, 34, 35, 37, 39, 40, 49, 57, 59, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 107, 108, 111, 122, 126, 129, 130, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 159, 183, 186, and 187. Exemplary residues may be those that are excluded from Site I and Site II, may be fully or partially solvent exposed, have minimal or no hydrogen-bonding interactions with nearby residues, may be minimally exposed to nearby reactive residues, and may be in regions that are highly flexible (e.g., C-D loop) or structurally rigid (e.g., B helix) as predicted by the three-dimensional crystal structure of hGH with its receptor.

[155] A wide variety of non-naturally encoded amino acids can be substituted for a given position in hGH. In general, a particular non-naturally encoded amino acid is selected for incorporation based on an examination of the three dimensional crystal structure of hGH with its receptor, a preference for conservative substitutions (i.e., aryl-based non-naturally encoded amino acids, such as p-acetylphenylalanine or O-propargyltyrosine substituting for Phe, Tyr or Trp), and the specific conjugation chemistry that one desires to introduce into the hGH polypeptide (e.g., the introduction of 4-azidophenylalanine if one wants to effect a Huisgen [3+2] cycloaddition with a water soluble polymer bearing an alkyne moiety or a amide bond formation with a water soluble polymer that bears an aryl ester that, in turn, incorporates a phosphine moiety).

[156] A subset of exemplary sites for incorporation of a non-naturally encoded amino acid include 33, 35, 37, 57, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 129, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 186, and 187. An examination of the crystal structure of hGH and its interactions with the hGH receptor indicates that the side chains of these amino acid residues are fully or partially accessible to

solvent and the side chain of a non-naturally encoded amino acid may point away from the protein surface and out into the solvent.

[157] Exemplary positions for incorporation of a non-naturally encoded amino acid include 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155. An examination of the crystal structure of hGH and its interactions with the hGH receptor indicates that the side chains of these amino acid residues are fully exposed to the solvent and the side chain of the native residue points out into the solvent.

[158] In some cases, the non-naturally encoded amino acid substitution(s) will be combined with other additions, substitutions or deletions within the hGH polypeptide to affect other biological traits of hGH. In some cases, the other additions, substitutions or deletions may increase the stability (e.g., resistance to proteolytic degradation) of the hGH polypeptide or increase affinity of the hGH polypeptide for a growth hormone receptor. In some embodiments, the hGH polypeptides comprise another addition, substitution or deletion that modulates affinity for the hGH receptor, modulates (e.g., increases or decreases) receptor dimerization, stabilizes receptor dimers, modulates circulating half-life, modulates release or bio-availabilty, facilitates purification, or improves or alters a particular route of administration. Similarly, hGH polypeptides can comprise protease cleavage sequences, reactive groups, antibody-binding domains (e.g., FLAG or poly-His) or other affinity based sequences (e.g., FLAG, poly-His, GST, etc.) or linked molecules (e.g., biotin) that improve detection (e.g., GFP), purification or other traits of the polypeptide.

[159] For instance, in addition to introducing one or more non-naturally encoded amino acids as set forth herein, one or more of the following substitutions are introduced: F10A, F10H or F10I; M14W, M14Q, or M14G; H18D; H21N; R167N; D171S; E174S; F176Y and I179T to increase the affinity of the hGH variant for its receptor.

[160] In some embodiments, the substitution of a non-naturally encoded amino acid generates an hGH antagonist. A subset of exemplary sites for incorporation of a non-naturally encoded amino acid include 1, 2, 3, 4, 5, 8, 9, 11, 12, 15, 16, 19, 22, 103, 109, 112, 113, 115, 116, 119, 120, 123, and 127. In other embodiments, the exemplary sites of incorporation of a non-naturally encoded amino acid include residues within the amino terminal region of helix A and a portion of helix C. In another embodiment, substitution of G120 with a non-naturally encoded amino acid such as p-azido-L-phenyalanine or O-propargyl-L-tyrosine. In other embodiments, the above-listed substitutions are combined with additional substitutions that cause the hGH polypeptide to be an hGH antagonist. For instance, a non-naturally encoded amino acid is substituted at one of the positions identified

herein and a simultaneous substitution is introduced at G120 (e.g., G120R, G120K, G120W, G120Y, G120F, or G120E).

[161] In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more amino acids are substituted with a non-naturally-encoded amino acid. In some cases, the hGH polypeptide further includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions of a non-naturally encoded amino acid for a naturally-occurring amino acid. For example, in some embodiments, at least two residues in the following regions of hGH are substituted with a non-naturally encoded amino acid: 1-5 (N-terminus); 32-46 (N-terminal end of the A-B loop); 97-105 (B-C loop); and 132-149 (C-D loop); and 184-191 (C-terminus). In some cases, the two or more non-naturally encoded residues are linked to one or more lower molecular weight linear or branched PEGs (approximately ~ 5-20 kDa in mass), thereby enhancing binding affinity and comparable serum half-life relative to the species attached to a single, higher molecular weight PEG.

[162] In some embodiments, up to two of the following residues are substituted with a non-naturally-encoded amino acid at position: 29, 30, 33, 34, 35, 37, 39, 40, 49, 57, 59, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 107, 108, 111, 122, 126, 129, 130, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 159, 183, 186, and 187. Thus, in some case, any of the following pairs of substitutions are made: K38X* and K140X*; K41X* and K145X*; Y35X* and D88X*; Y35X* and F92X*; Y35X* and Y143X*; F92X* and Y143X* wherein X* represents a non-naturally encoded amino acid. Preferred sites for incorporation of two or more non-naturally encoded amino acids include combinations of the following residues: 33, 35, 37, 57, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 129, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 186, and 187. Particularly preferred sites for incorporation of two or more non-naturally encoded amino acids include combinations of the following residues: 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155.

VII. Expression in Prokaryotes and Eukaryotes

[163] To obtain high level expression of a cloned hGH polynucleotide, one typically subclones polynucleotides encoding an hGH polypeptide of the invention into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.* and Ausubel *et al.*

[164] Bacterial expression systems for expressing hGH polypeptides of the invention are available in, e.g., *E. coli, Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In cases where orthogonal tRNAs and amino acyl tRNA synthetases (described above) are used to express the hGH polypeptides of the invention, host cells for expression are selected based on their ability to use the orthogonal components. Exemplary host cells include Gram-positive bacteria (e.g. *B. brevis* or *B. subtilis*, *Pseudomonas* or *Streptomyces*) and Gram-negative bacteria (*E. coli*), as well as yeast and other eukaryotic cells. Cells comprising O-tRNA/O-RS pairs can be used as described herein.

[165] Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[166] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of hGH-encoding polynucleotide in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding a hGH polypeptide of the invention and, if appropriate, signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[167] In addition to a promoter sequence, the expression cassette can also comprise a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[168] Methods for small-scale or large-scale fermentation can also be used in protein expression, including, e.g., fluidized bed bioreactors, hollow fiber bioreactors, roller bottle culture systems, and stirred tank bioreactor systems. Each of these methods can be performed in a batch, fed-batch, or continuous mode process.

[169] Human growth hormone polypeptides of the invention can generally be recovered using methods standard in the art. For example, culture medium or cell lysate can

be centrifuged or filtered to remove cellular debris. The supernatant is then typically concentrated or diluted to a desired volume or diafiltered into a suitable buffer to condition the preparation for further purification. Further purification of the hGH variant typically includes separating deamidated and clipped forms of the hGH variant from the intact form.

[170] Any of the following exemplary procedures can be employed for purification of an hGH polypeptides of the invention: affinity chromatography; anion- or cation-exchange chromatography (using, e.g., DEAE SEPHAROSE); chromatography on silica; reverse phase HPLC; gel filtration (using, e.g., SEPHADEX G-75); hydrophobic interaction chromatography; size-exclusion chromatography, metal-chelate chromatography; ultrafiltration/diafiltration; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; displacement chromatography; electrophoretic procedures (e.g. preparative isoelectric focusing), differential solubility (e.g. ammonium sulfate precipitation), SDS-PAGE, or extraction.

[171] Additional purification procedures include those described in U.S. Patent No. 4,612,367 and includes, e.g., (1) applying a mixture comprising an hGH polypeptide to a reverse phase macroporous acrylate ester copolymer resin support at a pH of from about 7 to about 9; and (2) eluting the hGH polypeptide from said support with an aqueous eluant having a pH of from about 7 to about 9 and containing from about 20% to about 80% by volume of an organic diluent selected from the group consisting of acetone, acetonitrile, and a combination of acetone and acetonitrile.

[172] Any of the following methods and procedures can be used to assess the yield and purity of an hGH protein that includes a non-naturally encoded amino acid: the Bradford assay, SDS-PAGE, mass spectrometry (e.g., MALDI-TOF) and other methods for characterizing proteins known to one skilled in the art.

VIII. Expression in Cell-Free Systems

[173] It may also be possible to obtain high level expression of an hGH polynucleotide using a cell-free (in-vitro) translational system. In these systems, which can include either mRNA as a template (in-vitro translation) or DNA as a template (combined in-vitro transcription and translation), the in vitro synthesis is directed by the ribosomes. Considerable effort has been applied to the development of cell-free protein expression systems. See, e.g., Kim, D.-M. and J.R. Swartz, *Biotechnology and Bioengineering*, 74 :309-316 (2001); Kim, D.-M. and J.R. Swartz, *Biotechnology Letters*, 22, 1537-1542, (2000); Kim, D.-M., and J.R. Swartz, *Biotechnology Progress*, 16, 385-390, (2000); Kim, D.-M., and J.R.

Swartz, *Biotechnology and Bioengineering*, 66, 180-188, (1999); and Patnaik, R. and J.R. Swartz, *Biotechniques* 24, 862-868, (1998). Another approach that may be applied to the expression of hGH polypeptides comprising a non-naturally encoded amino acid include the mRNA-peptide fusion technique. *See, e.g.,* R. Roberts and J. Szostak, *Proc. Natl Acad. Sci. (USA)* **94** 12297-12302 (1997); A. Frankel, *et al.*, *Chemistry & Biology* **10**, 1043-1050 (2003). In this approach, an mRNA template linked to puromycin is translated into peptide on the ribosome. If one or more tRNA molecules has been modified, non-natural amino acids can be incorporated into the peptide as well. After the last mRNA codon has been read, puromycin captures the C-terminus of the peptide. If the resulting mRNA-peptide conjugate is found to have interesting properties in an in vitro assay, its identity can be easily revealed from the mRNA sequence. In this way, one may screen libraries of hGH polypeptides comprising one or more non-naturally encoded amino acids to identify polypeptides having desired properties. More recently, in vitro ribosome translations with purified components have been reported that permit the synthesis of peptides substituted with non-naturally encoded amino acids. *See, e.g.,* A. Forster *et al.*, *Proc. Natl Acad. Sci. (USA)* **100** 6353 (2003).

IX. Macromolecular Polymers Coupled to hGH

[174] A variety of macromolecular polymers and other molecules can be linked to hGH polypeptides of the invention to improve biological properties of hGH. These macromolecular polymers can be linked to hGH via a naturally encoded amino acid or via a non-naturally enecoded amio acid.

[175] Water soluble polymers can be linked to the hGH polypeptides of the invention. The water soluble polymers are linked via a non-naturally encoded amino acid incorporated in the hGH polypeptide. Alternatively, the water soluble polymers are linked to an hGH polypeptide incorporating a non-naturally encoded amino acid via a naturally-occurring amino acid (e.g., cysteine, lysine or the amine group of the N-terminal residue). In some cases, the hGH polypeptides of the invention comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more non-naturally-encoded amino acid(s) linked to water soluble polymer(s) (e.g., PEG and/or oligosaccharides). In some cases, the hGH polypeptides of the invention further comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more naturally-encoded amino acid(s) linked to water soluble polymers. In some cases, the hGH polypeptides of the invention comprise one or more non-naturally encoded amino acid(s) linked to water soluble polymers and one or more naturally-occurring amino acids linked to water soluble polymers. In some embodiments, the

water soluble polymers used in the present invention enhance the serum half-life of the hGH polypeptide relative to the unconjugated form.

[176] The number of water soluble polymers linked to an hGH polypeptide (i.e., the extent of PEGylation or glycosylation) of the present invention can be adjusted to provide an altered (e.g., increased or decreased) *in vivo* half-life. In some embodiments, the half-life of hGH is increased at least about five-fold; preferably, at least about 10-fold; more preferably, at least about 50-fold; and most preferably, at least about 100-fold over an unmodified polypeptide.

[177] The water soluble polymer may be linear or branched. Typically, the water soluble polymer is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), but other water soluble polymers can also be employed. By way of example, PEG is used to describe this invention.

[178] PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods well known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, Vol. 3, pages 138-161). The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented as linked to hGH by the formula:

$$XO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}Y$$

where n is 2 to 10,000 and X is H or a terminal modification, e.g., a C_{1-4} alkyl.

[179] In some cases, a PEG used in the invention terminates on one end with hydroxy or methoxy, i.e., X is H or CH_3 ("methoxy PEG"). Alternatively, the PEG can terminate with a reactive group, thereby forming a bifunctional polymer. Typical reactive groups can include those reactive groups that are commonly used to react with the functional groups found in the 20 common amino acids (e.g., maleimide groups, activated carbonates (e.g., p-nitrophenyl ester), activated esters (e.g., N-hydroxysuccinimide, p-nitrophenyl ester) and aldehydes) as well as functional groups that are inert to the 20 common amino acids but that react specifically with complementary functional groups present in non-naturally encoded amino acids (e.g., azide groups, alkyne groups). It is noted that the other end of the PEG, which is shown in the above formula by Y, will attach either directly or indirectly to an hGH polypeptide via a naturally-occurring or non-naturally encoded amino acid. For instance, Y may be an amide, carbamate or urea linkage to an amine group (e.g., the epsilon amine of lysine or the *N*-terminus) of the polypeptide. Alternatively, Y may be a maleimide linkage to a thiol group (e.g., the thiol group of cysteine). Alternatively, Y may be a linkage

to a residue not commonly accessible via the 20 common amino acids. For example, an azide group on the PEG can be reacted with an alkyne group on the hGH polypeptide to form a Huisgen [3+2] cycloaddition product. Alternatively, an alkyne group on the PEG can be reacted with an azide group present in a non-naturally encoded amino acid to form a similar product. In some embodiments, a strong nucleophile (e.g., hydrazine, hydrazide, hydroxylamine, semicarbazide) can be reacted with an aldehyde or ketone group present in a non-naturally encoded amino acid to form a hydrazone, oxime or semicarbazone, as applicable, which in some cases can be further reduced by treatment with an appropriate reducing agent. Alternatively, the strong nucleophile can be incorporated into the hGH polypeptide via a non-naturally encoded amino acid and used to react preferentially with a ketone or aldehyde group present in the water soluble polymer.

[180] Any molecular mass for a PEG can be used as practically desired, e.g., from about 1,000 Daltons (Da) to 100,000 Da (e.g., sometimes 1-50 kDa or 10-40 kDa.). Branched chain PEGs, e.g., with each chain having a MW ranging from 10-40 kDa (e.g., 5-20 kDa) can also be used. A wide range of PEG molecules are described in, e.g., the 2000 Shearwater Polymers, Inc. catalog, incorporated herein by reference.

[181] Generally, at least one terminus of the PEG molecule is activated for reaction with the non-naturally-encoded amino acid. For example, PEG derivatives bearing alkyne and azide moieties for reaction with amino acid side chains can be used to attach PEG to non-naturally encoded amino acids as described herein. If the non-naturally encoded amino acid comprises an azide, then the PEG will typically contain either an alkyne moiety to effect formation of the [3+2] cycloaddition product or an activated PEG species (i.e., ester, carbonate) containing a phosphine group to effect formation of the amide linkage. Alternatively, if the non-naturally encoded amino acid comprises an alkyne, then the PEG will typically contain an azide moiety to effect formation of the [3+2] Huisgen cycloaddition product. If the non-naturally encoded amino acid comprises a carbonyl group, the PEG will typically comprise a potent nucleophile (e.g., a hydrazide, hydroxylamine or semicarbazide functionality) in order to effect formation of corresponding hydrazone, oxime, and semicarbazone linkages, respectively. In other alternatives, a reverse of the orientation of the reactive groups described above can be used, i.e., an azide moiety in the non-naturally encoded amino acid can be reacted with a PEG derivative containing an alkyne.

[182] In some embodiments, the hGH variant with a PEG derivative contains a chemical functionality that is reactive with the chemical functionality present on the side chain of the non-naturally encoded amino acid.

PEG derivatives containing a strong nucleophilic group (i.e., hydrazide, hydrazine, hydroxylamine or semicarbazide)

[183] In one embodiment of the invention, an hGH polypeptide comprising a carbonyl-containing non-naturally encoded amino acid is modified with a PEG derivative that contains a terminal hydrazine, hydroxylamine, hydrazide or semicarbazide moiety that is linked directly to the PEG backbone.

[184] In some embodiments, the hydroxylamine-terminal PEG derivative will have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}O\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

[185] In some embodiments, the hydrazine- or hydrazide-containing PEG derivative will have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}X\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 and X is optionally a carbonyl group (C=O) that can be present or absent.

[186] In some embodiments, the semicarbazide-containing PEG derivative will have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{ -}O\text{-}(CH_2)_m\text{-}NH\text{-}C(O)\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000.

[187] In another embodiment of the invention, an hGH polypeptide comprising a carbonyl-containing amino acid is modified with a PEG derivative that contains a terminal hydroxylamine, hydrazide or semicarbazide moiety that is linked to the PEG backbone by means of an amide linkage.

[188] In some embodiments, the hydroxylamine-terminal PEG derivatives have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)(CH_2)_m\text{-}O\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

[189] In some embodiments, the hydrazine- or hydrazide-containing PEG derivatives have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)(CH_2)_m\text{-}X\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, n is 100-1,000 and X is optionally a carbonyl group (C=O) that can be present or absent.

[190] In some embodiments, the semicarbazide-containing PEG derivatives have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)(CH_2)_m\text{-}NH\text{-}C(O)\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000.

[191] In another embodiment of the invention, an hGH polypeptide comprising a carbonyl-containing amino acid is modified with a branched PEG derivative that contains a terminal hydrazine, hydroxylamine, hydrazide or semicarbazide moiety, with each chain of the branched PEG having a MW ranging from 10-40 kDa and, more preferably, from 5-20 kDa.

[192] In another embodiment of the invention, an hGH polypeptide comprising a non-naturally encoded amino acid is modified with a PEG derivatives having a branched structure. For instance, in some embodiments, the hydrazine- or hydrazide-terminal PEG derivative will have the following structure:

$[RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)]_2CH(CH_2)_m\text{-}X\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000, and X is optionally a carbonyl group (C=O) that can be present or absent.

[193] In some embodiments, the PEG derivatives containing a semicarbazide group will have the structure:

$[RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}C(O)\text{-}NH\text{-}CH_2\text{-}CH_2]_2CH\text{-}X\text{-}(CH_2)_m\text{-}NH\text{-}C(O)\text{-}NH\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), X is optionally NH, O, S, C(O) or not present, m is 2-10 and n is 100-1,000.

[194] In some embodiments, the PEG derivatives containing a hydoxylamine group will have the structure:

$[RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}C(O)\text{-}NH\text{-}CH_2\text{-}CH_2]_2CH\text{-}X\text{-}(CH_2)_m\text{-}O\text{-}NH_2$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), X is optionally NH, O, S, C(O) or not present, m is 2-10 and n is 100-1,000.

[195] The degree and sites at which the water soluble polymer(s) are linked to hGH can modulate the binding of hGH to the hGH receptor at Site 1. In some embodiments, the linkages are arranged such that the hGH polypeptide binds the hGH receptor at Site 1 with a K_d of about 400 nM or lower, with a K_d of 150 nM or lower, and in some cases with a K_d of 100 nM or lower, as measured by an equilibrium binding assay, such as that described in Spencer *et al.*, *J. Biol. Chem.*, 263:7862-7867 (1988).

[196] Methods and chemistry for activation of polymers as well as for conjugation of peptides are described in the literature. Commonly used methods for activation of polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, *etc.* (*see*, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTAL AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

[197] Several reviews and monographs on the functionalization and conjugation of PEG are available. *See,* for example, Harris, *Macronol. Chem. Phys.* C25: 325-373 (1985); Scouten, *Methods in Enzymology* 135: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* 14: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995).

[198] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and enzymes *e.g.* Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* **11**: 141-45 (1985)).

[199] PEGylation (i.e., addition of any water soluble polymer) of hGH polypeptides containing a non-naturally encoded amino acid, such as *p*-azido-L-phenylalanine, is carried out by any convenient method. For example, hGH polypeptide are PEGylated with an alkyne-terminated mPEG derivative. Briefly, an excess of solid mPEG(5000)-O-CH_2-C≡CH is added, with stirring, to an aqueous solution of *p*-azido-L-Phe-containing hGH at room temperature. Typically, the aqueous solution is buffered with a buffer having a pK_a near the pH at which the reaction is to be carried out (generally about pH 4-10). Examples of suitable buffers for PEGylation at pH 7.5, for instance, include HEPES, phosphate, borate, Tris-HCl, EPPS, and TES. The pH is continuously monitored and adjusted if necessary. The reaction is typically allowed to continue for between 1-48 hours.

[200] The reaction products are subsequently subjected to hydrophobic interaction chromatography to separate the PEGylated hGH variants from free mPEG(5000)-O-CH_2-C≡CH and any high-molecular weight complexes of the pegylated hGH polypeptide which may form when unblocked PEG is activated at both ends of the molecule, thereby crosslinking hGH variant molecules. The conditions during hydrophobic interaction chromatography are such that free mPEG(5000)-O-CH_2-C≡CH flows through the column, while any crosslinked PEGylated hGH variant complexes elute after the desired forms, which contain one hGH variant molecule conjugated to one or more PEG groups. Suitable conditions vary depending on the relative sizes of the cross-linked complexes versus the desired conjugates and are readily determined by those skilled in the art. The eluent containing the desired conjugates is concentrated by ultrafiltration and desalted by diafiltration.

[201] If necessary, the PEGylated hGH obtained from the hydrophobic chromatography can be purified further by one or more of the following procedures: affinity chromatography; anion- or cation-exchange chromatography (using, e.g., DEAE SEPHAROSE); chromatography on silica; reverse phase HPLC; gel filtration (using, e.g., SEPHADEX G-75); hydrophobic interaction chromatography; size-exclusion chromatography, metal-chelate chromatography; ultrafiltration/diafiltration; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; displacement chromatography; electrophoretic procedures (e.g. preparative isoelec- tric focusing), differential solubility (e.g. ammonium sulfate precipitation), or extraction. Apparent molecular weight may be estimated by GPC by comparison to globular protein standards (PROTEIN PURIFICATION METHODS, A PRACTICAL APPROACH (Harris & Angal, Eds.) IRL Press 1989, 293-306). The purity of the hGH-PEG conjugate can be assessed by proteolytic degradation (e.g., trypsin cleavage) followed by mass spectrometry analysis. Pepinsky B., *et.al., J. Pharmcol. & Exp. Ther.* 297(3):1059-66 (2001).

[202] A water soluble polymer linked to an amino acid of an hGH polypeptide of the invention can be further derivatized or substituted without limitation.

Azide-containing PEG derivatives

[203] In another embodiment of the invention, an hGH polypeptide is modified with a PEG derivative that contains an azide moiety that will react with an alkyne moiety present on the side chain of the non-naturally encoded amino acid. In general, the

PEG derivatives will have an average molecular weight ranging from 1-50 kDa and, more preferably, from 10-40 kDa.

[204] In some embodiments, the azide-terminal PEG derivative will have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}N_3$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

[205] In another embodiment, the azide-terminal PEG derivative will have the structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}NH\text{-}C(O)\text{-}(CH_2)_p\text{-}N_3$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

[206] In another embodiment of the invention, an hGH polypeptide comprising a alkyne-containing amino acid is modified with a branched PEG derivative that contains a terminal azide moiety, with each chain of the branched PEG having a MW ranging from 10-40 kDa and, more preferably, from 5-20 kDa. For instance, in some embodiments, the azide-terminal PEG derivative will have the following structure:

$[RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)]_2CH(CH_2)_m\text{-}X\text{-}(CH_2)_pN_3$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10, and n is 100-1,000, and X is optionally an O, N, S or carbonyl group (C=O), in each case that can be present or absent.

Alkyne-containing PEG derivatives

[207] In another embodiment of the invention, an hGH polypeptide is modified with a PEG derivative that contains an alkyne moiety that will react with an azide moiety present on the side chain of the non-naturally encoded amino acid.

[208] In some embodiments, the alkyne-terminal PEG derivative will have the following structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}C{\equiv}CH$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

[209] In another embodiment of the invention, an hGH polypeptide comprising an alkyne-containing non-naturally encoded amino acid is modified with a PEG

derivative that contains a terminal azide or terminal alkyne moiety that is linked to the PEG backbone by means of an amide linkage.

[210] In some embodiments, the alkyne-terminal PEG derivative will have the following structure:

$RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}NH\text{-}C(O)\text{-}(CH_2)_p\text{-}C{\equiv}CH$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10 and n is 100-1,000.

[211] In another embodiment of the invention, an hGH polypeptide comprising an azide-containing amino acid is modified with a branched PEG derivative that contains a terminal alkyne moiety, with each chain of the branched PEG having a MW ranging from 10-40 kDa and, more preferably, from 5-20 kDa. For instance, in some embodiments, the alkyne-terminal PEG derivative will have the following structure:

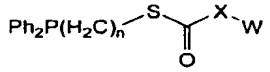
$[RO\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)]_2CH(CH_2)_m\text{-}X\text{-}(CH_2)_p\ C{\equiv}CH$

where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10, and n is 100-1,000, and X is optionally an O, N, S or carbonyl group (C=O), or not present.

Phosphine-containing PEG derivatives

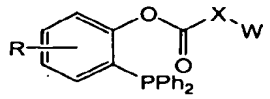
[212] In another embodiment of the invention, an hGH polypeptide is modified with a PEG derivative that contains an activated functional group (e.g., ester, carbonate) further comprising an aryl phosphine group that will react with an azide moiety present on the side chain of the non-naturally encoded amino acid. In general, the PEG derivatives will have an average molecular weight ranging from 1-50 kDa and, more preferably, from 10-40 kDa.

[213] In some embodiments, the PEG derivative will have the structure:



wherein n is 1-10; X can be O, N, S or not present, Ph is phenyl, and W is a water soluble polymer.

[214] In some embodiments, the PEG derivative will have the structure:



wherein X can be O, N, S or not present, Ph is phenyl, W is a water soluble polymer and R can be H, alkyl, aryl, substituted alkyl and substituted aryl groups. Exemplary R groups include but are not limited to $-CH_2$, $-C(CH_3)_3$, -OR', -NR'R", -SR', -halogen, -C(O)R', -

CONR'R", -$S(O)_2$R', -$S(O)_2$NR'R", -CN and $-NO_2$. R', R", R"' and R"" each independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, *e.g.*, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, -CF_3 and $-CH_2CF_3$) and acyl (*e.g.*, -$C(O)CH_3$, -$C(O)CF_3$, -$C(O)CH_2OCH_3$, and the like).

Other PEG derivatives and General PEGylation techniques

[215] Other exemplary PEG molecules that can be linked to hGH polypeptides, as well as PEGylation methods include those described in, e.g., U.S. Patent Publication No. 2004/0001838, U.S. Patent Publication No. 2002/0052009, U.S. Patent Publication No. 2003/0162949, U.S. Patent No. 6,646,110, U.S. Patent No. 5,824,778, U.S. Patent No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Patent No. 4,902,502, U.S. Patent No. 5,281,698, U.S. Patent No.. 5,122,614, U.S. Patent No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Patent No. 5,736,625, WO 98/05363, EP 809 996, U.S. Patent No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Patent No. 5,473,034, U.S. Patent No. 5,516,673, EP 605 963, U.S. Patent No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

Enhancing affinity for serum albumin

[216] Various molecules can also be fused to the hGH polypeptides of the invention to promote the half-life of hGH in serum. In some embodiments, molecules are linked or fused to hGH polypeptides of the invention to enhance affinity for endogenous serum albumin in an animal.

[217] For example, in some cases, a recombinant fusion of an hGH polypeptide and an albumin binding sequence is made. Exemplary albumin binding sequences include, e.g., the albumin binding domain from streptococcal protein G (*see. e.g.*, Makrides *et al.*, *J. Pharmacol. Exp. Ther.* 277:534-542 (1996) and Sjolander *et al.*, *J. Immunol. Methods* 201:115-123 (1997)), or albumin-binding peptides such as those described in, e.g., Dennis, *et al.*, *J. Biol. Chem.* 277:35035-35043 (2002).

[218] In other embodiments, the hGH polypeptides of the invention are acylated with fatty acids. In some cases, the fatty acids promote binding to serum albumin. *See, e.g.*, Kurtzhals, *et al.*, *Biochem. J.* 312:725-731 (1995).

[219] In other embodiments, the hGH polypeptides of the invention are fused directly with serum albumin (e.g., human serum albumin). *See, e.g.*, Syed, *et al.*, *Blood* 89:3243-3252 (1997) and Yeh *et al.*, *Proc. Natl. Acad. Sci. USA* 89:1904-1908 (1992).

[220] Those of skill in the art will recognize that other molecules can also be linked to hGH to promote binding to serum albumin or other serum components.

X Glycosylation of hGH

[221] The invention includes hGH polypeptides incorporating one or more non-naturally encoded amino acids bearing saccharide residues. The saccharide residues may be either natural (e.g., N-acetylglucosamine) or non-natural (e.g., 3-fluorogalactose). The saccharides may be linked to the non-naturally encoded amino acids either by an N- or O-linked glycosidic linkage (e.g., N-acetylgalactose-L-serine) or a non-natural linkage (e.g., an oxime or the corresponding C- or S-linked glycoside).

[222] The saccharide (e.g., glycosyl) moieties can be added to hGH polypeptides either *in vivo* or *in vitro*. In some embodiments of the invention, an hGH polypeptide comprising a carbonyl-containing non-naturally encoded amino acid is modified with a saccharide derivatized with an aminooxy group to generate the corresponding glycosylated polypeptide linked via an oxime linkage. Once attached to the non-naturally encoded amino acid, the saccharide may be further elaborated by treatment with glycosyltransferases and other enzymes to generate an oligosaccharide bound to the hGH polypeptide. *See, e.g.*, H. Liu, et al. *J. Am. Chem. Soc.* 125: 1702-1703 (2003).

[223] In some embodiments of the invention, an hGH polypeptide comprising a carbonyl-containing non-naturally encoded amino acid is modified directly with a glycan with defined structure prepared as an aminooxy derivative. One skilled in the art

will recognize that other functionalities, including azide, alkyne, hydrazide, hydrazine, and semicarbazide, can be used to link the saccharide to the non-naturally encoded amino acid.

[224] In some embodiments of the invention, an hGH polypeptide comprising an azide or alkynyl-containing non-naturally encoded amino acid can then be modified by, e.g., a Huisgen [3+2] cycloaddition reaction with, e.g., alkynyl or azide derivatives, respectively. This method allows for proteins to be modified with extremely high selectivity.

XI. GH Supergene Family Member Dimers

[225] The present invention also provides for GH supergene family member (including but not limited to hGH) dimers or multimers (i.e., trimers, tetramers, etc.) where a GH supergene family member polypeptide containing one or more non-naturally encoded amino acids is bound to another GH supergene family member variant either directly or via a linker. Due to its increased molecular weight compared to monomers, GH supergene family member dimer conjugates may exhibit an increased plasma half-life relative to monomeric GH supergene family member. In some embodiments, the GH supergene family member dimers of the invention will allow for enhanced dimerization of the GH supergene family member receptor. In other embodiments, the GH supergene family member dimers of the invention will act as an GH supergene family member receptor antagonist.

[226] In some embodiments, each of the hGH molecules present in an hGH dimer comprises a non-naturally encoded amino acid linked to a water soluble polymer that is present within the Site II binding region. As such, each of the hGH molecules of the dimer are accessible for binding to the hGH receptor via the Site I interface but are unavailable for binding to a second hGH receptor via the Site II interface. Thus, the hGH dimer can engage the Site I binding sites of each of two distinct hGH receptors but, as the hGH molecules have a water soluble polymer attached to a non-genetically encoded amino acid present in the Site II region, the hGH receptors cannot engage the Site II region of the hGH ligand and the dimer acts as an hGH antagonist.

[227] In some embodiments, the GH supergene family member polypeptides are linked directly, e.g., via an Asn-Lys amide linkage or Cys-Cys disulfide linkage. In some embodiments, the two linked GH supergene family member polypeptides will comprise different non-naturally encoded amino acids to facilitate dimerization, e.g., an alkyne in one non-naturally encoded amino acid of a first hGH polypeptide and an azide in a second non-naturally encoded amino acid of a second GH supergene family member polypeptide will be

conjugated via a Huisgen [3+2] cycloaddition. Alternatively, a first GH supergene family member polypeptide comprising a ketone-containing non-naturally encoded amino acid can be conjugated to a second GH supergene family member polypeptide comprising a hydroxylamine-containing non-naturally encoded amino acid and the two GH supergene family member polypeptides are reacted via formation of the corresponding oxime.

[228] Alternatively, the two GH supergene family member polypeptides are linked via a linker. Any hetero- or homo-bifunctional linker can be used to link the two GH supergene family member polypeptides, which can have the same or different primary sequence. In some cases, the linker used to tether the two GH supergene family member variants together can be a bifunctional PEG reagent.

[229] In some embodiments, the invention provides water-soluble bifunctional linkers that have a dumbbell structure that includes: a) an azide, an alkyne, a hydrazine, a hydrazide, a hydroxylamine, or a carbonyl-containing moiety on at least a first end of a polymer backbone; and b) at least a second functional group on a second end of the polymer backbone. The second functional group can be the same or different as the first functional group. The second functional group, in some embodiments, is not reactive with the first functional group. The invention provides, in some embodiments, water-soluble compounds that comprise at least one arm of a branched molecular structure. For example, the branched molecular structure can be dendritic.

[230] In some embodiments, the invention provides GH supergene family member multimers formed by reactions with water soluble activated polymers that have the structure:

$R\text{-}(CH_2CH_2O)_n\text{-}O\text{-}(CH_2)_m\text{-}X$

wherein n is from about 5 to 3,000, m is 2-10, X can be an azide, an alkyne, a hydrazine, a hydrazide, a hydroxylamine, or carbonyl-containing moiety, and R is a capping group, a functional group, or a leaving group that can be the same or different as X. R can be, for example, a functional group selected from the group consisting of hydroxyl, protected hydroxyl, alkoxyl, N-hydroxysuccinimidyl ester, 1-benzotriazolyl ester, N-hydroxysuccinimidyl carbonate, 1-benzotriazolyl carbonate, acetal, aldehyde, aldehyde hydrates, alkenyl, acrylate, methacrylate, acrylamide, active sulfone, amine, aminooxy, protected amine, hydrazide, protected hydrazide, protected thiol, carboxylic acid, protected carboxylic acid, isocyanate, isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxals, diones, mesylates, tosylates, and tresylate, alkene, and ketone.

XII. Measurement of hGH Activity and Affinity of hGH for the hGH Receptor

[231] The hGH receptor can be prepared as described in McFarland *et al.*, *Science*, 245: 494-499 (1989) and Leung, D., *et al.*, *Nature*, 330:537-543 (1987). hGH polypeptide activity can be determined using standard *in vitro* or *in vivo* assays. For example, cell lines that proliferate in the presence of hGH (e.g., a cell line expressing the hGH receptor or a lactogenic receptor) can be used to monitor hGH receptor binding. *See, e.g.*, Clark, R., *et al.*, *J. Biol. Chem.* 271(36):21969 (1996); Wada, *et al.*, *Mol. Endocrinol.* 12:146-156 (1998); Gout, P. W., *et al. Cancer Res.* 40, 2433-2436 (1980); WO 99/03887. For a non-PEGylated hGH polypeptide comprising a non-natural amino acid, the affinity of the hormone for its receptor can be measured by using a BIAcore™ biosensor (Pharmacia). *See, e.g.*, U.S. Patent No. 5,849,535; Spencer, S. A., *et al.*, *J. Biol. Chem.*, 263:7862-7867 (1988). *In vivo* animal models for testing hGH activity include those described in, e.g., Clark *et al.*, *J. Biol. Chem.* 271(36):21969-21977 (1996). Assays for dimerization capability of hGH polypeptides comprising one or more non-naturally encoded amino acids can be conducted as described in Cunningham, B., *et al.*, *Science*, 254:821-825 (1991) and Fuh, G., *et al.*, *Science*, 256:1677-1680 (1992).

XIII. Measurement of Potency, Functional In Vivo Half-Life, and Pharmacokinetic Parameters

[232] The potency and functional in vivo half-life of an hGH polypeptide comprising a non-naturally encoded amino acid can be determined according to the protocol described in Clark, R., *et al.*, *J. Biol. Chem.* 271, 36, 21969-21977 (1996).

[233] Pharmacokinetic parameters for an hGH polypeptide comprising a non-naturally encoded amino acid can be evaluated in normal Sprague-Dawley male rats (N=5 animals per treatment group). Animals will receive either a single dose of 25 ug/rat iv or 50 ug/rat sc, and approximately 5-7 blood samples will be taken according to a predefined time course, generally covering about 6 hours for an hGH polypeptide comprising a non-naturally encoded amino acid not conjugated to a water soluble polymer and about 4 days for an hGH polypeptide comprising a non-naturally encoded amino acid and conjugated to a water soluble polymer. Pharmacokinetic data for hGH is well-studied in several species and can be compared directly to the data obtained for hGH comprising a non-naturally encoded amino acid. *See* Mordenti J., *et al.*, *Pharm. Res.* 8(11):1351-59 (1991).

XIV. Administration and Pharmaceutical Compositions

[234] Human growth hormone polypeptides of the invention can be administered directly to a mammalian subject. Administration is by any of the routes normally used for introducing hGH to a subject. The hGH polypeptide compositions according to embodiments of the present invention include those suitable for oral, rectal, topical, inhalation (e.g., via an aerosol), buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular, intradermal, intraarticular, intrapleural, intraperitoneal, inracerebral, intraarterial, or intravenous), topical (i.e., both skin and mucosal surfaces, including airway surfaces) and transdermal administration, although the most suitable route in any given case will depend on the nature and severity of the condition being treated. Administration can be either local or systemic. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials. hGH polypeptides of the invention can be prepared in a mixture in a unit dosage injectable form (e.g., solution, suspension, or emulsion) with a pharmaceutically acceptable carrier. hGH polypeptides of the invention can also be administered by continuous infusion (using, e.g., minipumps such as osmotic pumps), single bolus or slow-release depot formulations.

[235] Formulations suitable for administration include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[236] The pharmaceutical compositions of the invention may comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions (including optional pharmaceutically acceptable carriers, excipients, or stabilizers) of the present invention (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed. 1985)).

[237] Suitable carriers include buffers containing phosphate, borate, HEPES, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other

carbohydrates, including glucose, mannose, or dextrins; chelating agents such as EDTA; divalent metal ions such as zinc, cobalt, or copper; sugar alcohols such as mannitol or sorbitol; salt-forming counter ions such as sodium; and/or nonionic surfactants such as Tween™, Pluronics™, or PEG.

[238] hGH polypeptides of the invention, including those linked to water soluble polymers such as PEG can also be administered by or as part of sustained-release systems. Sustained-release compositions include, e.g., semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Sustained-release matrices include from biocompatible materials such as poly(2-hydroxyethyl methacrylate) (Langer *et al.*, *J. Biomed. Mater. Res.*, 15: 167-277 (1981); Langer, *Chem. Tech.*, 12: 98-105 (1982), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988), polylactides (polylactic acid) (U.S. Patent No. 3,773,919; EP 58,481), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (copolymers of lactic acid and glycolic acid) polyanhydrides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (U. Sidman *et al.*, *Biopolymers*, 22, 547-556 (1983), poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. Sustained-release compositions also include a liposomally entrapped compound. Liposomes containing the compound are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 82: 3688-3692 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appln. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324.

[239] Liposomally entrapped hGH polypeptides can be prepared by methods described in, e.g., DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 82: 3688-3692 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appln. 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Composition and size of liposomes are well known or able to be readily determined empirically by one skilled in the art. Some examples of liposomes asdescribed in, e.g., Park JW, *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1327-1331 (1995); Lasic D and Papahadjopoulos D (eds): MEDICAL APPLICATIONS OF LIPOSOMES (1998); Drummond DC, *et al.*, Liposomal drug delivery systems for cancer therapy, in Teicher B (ed): CANCER DRUG DISCOVERY AND DEVELOPMENT (2002); Park JW, *et al.*, *Clin.*

Cancer Res. 8:1172-1181 (2002); Nielsen UB, *et al.*, *Biochim. Biophys. Acta* 1591(1-3):109-118 (2002); Mamot C, *et al.*, *Cancer Res.* 63: 3154-3161 (2003).

[240] The dose administered to a patient in the context of the present invention should be sufficient to effect a beneficial response in the subject over time. Generally, the total pharmaceutically effective amount of the hGH variant administered parenterally per dose is in the range of about 0.05 mg/kg/day to about 0.5 mg/kg/day of patient body weight, although this is subject to therapeutic discretion. Generally, a PEGylated hGH polypeptide of the invention can be administered by any of the routes of administration described above. However, it is likely that a PEGylated hGH polypeptide need not be administered as frequently as a non-PEGylated hGH variant.

XV. Therapeutic Uses of hGH Polypeptides of the Invention

[241] The hGH polypeptides of the invention are useful for treating a wide range of disorders.

[242] The hGH agonist polypeptides of the invention are useful, for example, for treating growth deficiency, immune disorders, and for stimulating heart function. Individuals with growth deficiencies include, e.g., individuals with Turner's Syndrome, GH-deficient individuals (including children), children who experience a slowing or retardation in their normal growth curve about 2-3 years before their growth plate closes (sometimes known as "short normal children"), and individuals where the insulin-like growth factor-I (IGF-I) response to GH has been blocked chemically (i.e., by glucocorticoid treatment) or by a natural condition such as in adult patients where the IGF-I response to GH is naturally reduced.

[243] An agonist hGH variant can act to stimulate the immune system of a mammal by increasing its immune function, whether the increase is due to antibody mediation or cell mediation, and whether the immune system is endogenous to the host treated with the hGH polypeptide or is transplanted from a donor to the host recipient given the hGH polypeptide (as in bone marrow transplants). "Immune disorders" include any condition in which the immune system of an individual has a reduced antibody or cellular response to antigens than normal, including those individuals with small spleens with reduced immunity due to drug (e.g., chemotherapeutic) treatments. Examples individuals with immune disorders include, e.g., elderly patients, individuals undergoing chemotherapy or radiation therapy, individuals recovering from a major illness, or about to undergo surgery, individuals with AIDS, Patients with congenital and acquired B-cell deficiencies such as

hypogammaglobulinemia, common varied agammaglobulinemia, and selective immunoglobulin deficiencies (e.g., IgA deficiency, patients infected with a virus such as rabies with an incubation time shorter than the immune response of the patient; and individuals with hereditary disorders such as diGeorge syndrome.

[244] hGH antagonist polypeptides of the invention are useful for the treatment of gigantism and acromegaly, diabetes and complications (diabetic retinopathy, diabetic neuropathy) arising from diabetes, vascular eye diseases (e.g., involving proliferative neovascularization), nephropathy, and GH-responsive malignancies.

[245] Vascular eye disease include, e.g., retinopathy (caused by, e.g., prematurity or sickle cell anemia) and macular degeneration.

[246] GH-responsive malignancies include, e.g., Wilm's tumor, sarcomas (e.g., osteogenic sarcoma), breast, colon, prostate, and thyroid cancer, and cancers of tissues that express GH receptor mRNA (i.e., placenta, thymus, brain, salivary gland, prostate, bone marrow, skeletal muscle, trachea, spinal cord, retina, lymph node and from Burkitt's lymphoma, colorectal carcinoma, lung carcinoma, lymphoblastic leukemia, and melanoma).

EXAMPLES

[247] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

[248] This example describes one of the many potential sets of criteria for the selection of preferred sites of incorporation of non-naturally encoded amino acids into hGH.

[249] This Example demonstrates how preferred sites within the hGH polypeptide were selected for introduction of a non-naturally encoded amino acid. The crystal structure 3HHR, composed of hGH complexed with two molecules of the extracellular domain of receptor (hGHbp), was used to determine preferred positions into which one or more non-naturally encoded amino acids could be introduced. Other hGH structures (e.g. 1AXI) were utilized to examine potential variation of primary and secondary structural elements between crystal structure datasets. The coordinates for these structures are available from the Protein Data Bank (PDB) (Berstein et al. *J. Mol. Biol.* 1997, 112, pp 535) or via The Research Collaboratory for Structural Bioinformatics PDB at http://www.rcsb.org. The structural model 3HHR contains the entire mature 22 kDa sequence of hGH with the exception of residues 148 – 153 and the C-terminal F191 residue which were omitted due to disorder in the crystal. Two disulfide bridges are present, formed by C53 and C165 and C182 and C185.

[250] Sequence numbering used in this example is according to the amino acid sequence of mature hGH (22 kDa variant) shown in SEQ ID NO:2.

[251] The following criteria were used to evaluate each position of hGH for the introduction of a non-naturally encoded amino acid: the residue (a) should not interfere with binding of either hGHbp based on structural analysis of 3HRR, 1AXI, and 1HWG (crystallographic structures of hGH conjugated with hGHpb monomer or dimer), b) should not be affected by alanine scanning mutagenesis (Cunningham et al. *Science*1989, 244, pp1081), (c) should be surface exposed and exhibit minimal van der Waals or hydrogen bonding interactions with surrounding residues, (d) should be either deleted or variable in hGH variants (e.g. Tyr35, Lys38, Phe92, Lys140), (e) would result in conservative changes

upon substitution with a non-naturally encoded amino acid and (f) could be found in either highly flexible regions (e.g. CD loop) or structurally rigid regions (e.g. Helix B). In addition, further calculations were performed on the hGH molecule, utilizing the Cx program (Pintar et al. *Bioinformatics*, 18, pp 980) to evaluate the extent of protrusion for each protein atom. As a result, in some embodiments, the non-naturally encoded encoded amino acid is substituted at, but not limited to, one or more of the following positions of hGH: before position 1 (i.e., at the N-terminus), 1, 2, 3, 4, 5, 9, 11, 12, 15, 16, 19, 22, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,43, 44, 45, 46, 47, 48, 49, 52, 55, 57, 59, 65, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 111, 112, 113, 115, 116, 119, 120, 122, 123, 126, 127, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 158, 159, 161, 168, 172, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192 (i.e., at the carboxyl terminus of the protein) or combinations thereof.

[252] Some sites for generation of an hGH antagonist include: before position 1 (i.e., at the N-terminus), 1, 2, 3, 4, 5, 8, 9, 11, 12, 15, 16, 19, 22, 103, 109, 112, 113, (115), 116, 119, 120, 123, and 127. These sites were chosen utilizing criteria c – e of the agonist design. The antagonist design may also include site-directed modifications of site 1 residues to increase binding affinity to hGHbp.

Example 2

[253] This example details expression of a modified hGH polypeptide in *E. coli*.

[254] This Example demonstrates how an hGH polypeptide including a non-naturally encoded amino acid can be expressed in *E. coli*. An introduced translation system that comprises an orthogonal tRNA (O-tRNA) and an orthogonal aminoacyl tRNA synthetase (O-RS) is used. The O-RS preferentially aminoacylates the O-tRNA with a non-naturally encoded amino acid. In turn the translation system inserts the non-naturally encoded amino acid into hGH, in response to an encoded selector codon. The following O-RS and O-tRNA sequences are used:

SEQ ID NO:4	*M. jannaschii* $mtRNA^{Tyr}_{CUA}$	tRNA
SEQ ID NO:5	*HLAD03; an optimized amber supressor tRNA*	tRNA
SEQ ID NO:6	*HL325A; an optimized AGGA frameshift supressor tRNA*	tRNA

SEQ ID NO:7	*Aminoacyl tRNA synthetase for the incorporation of p-azido-L-phenylalanine* *p-Az-PheRS(6)*	RS
SEQ ID NO:8	*Aminoacyl tRNA synthetase for the incorporation of p-benzoyl-L-phenylalanine* *p-BpaRS(1)*	RS
SEQ ID NO:9	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS
SEQ ID NO:10	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS
SEQ ID NO:11	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS
SEQ ID NO:12	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(1)*	RS
SEQ ID NO:13	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(3)*	RS
SEQ ID NO:14	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(4)*	RS
SEQ ID NO:15	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(2)*	RS
SEQ ID NO:16	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW1)*	RS
SEQ ID NO:17	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW5)*	RS
SEQ ID NO:18	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW6)*	RS

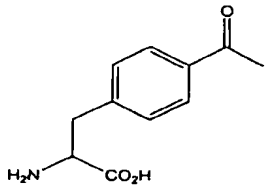
SEQ ID NO:19	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (AzPheRS-5)*	RS
SEQ ID NO:20	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (AzPheRS-6)*	RS

[255] The transformation of *E. coli* with plasmids containing the modified hGH gene and the orthogonal aminoacyl tRNA synthetase/tRNA pair (specific for the desired non-naturally encoded amino acid) allows the site-specific incorporation of non-naturally encoded amino acid into the hGH polypeptide. The transformed *E. coli*, grown at 37° C in media containing between 0.01 – 100 mM of the particular non-naturally encoded amino acid, will then express modified hGH with high fidelity and efficiency. Methods for purification of hGH are well known in the art (Panda, A. *Adv. Biochem Engin/Biotechnol* 2003, 85, p43) and will be confirmed by SDS-PAGE, Western Blot analyses, and electrospray-ionization ion trap mass spectrometry.

Example 3

[256] This example details introduction of a carbonyl-containing amino acid and subsequent reaction with a hydroxylamine-containing PEG.

[257] This Example example demonstrates a method for the generation of an hGH polypeptide that incorporates a ketone-containing non-naturally encoded amino acid that is subsequently reacted with a hydroxylamine-containing PEG of approximately 5,000 MW. Each of the residues 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155 is substituted with a non-naturally encoded amino acid having the following structure:



[258] The sequences utilized for site-specific incorporation of p-acetyl-phenylalanine into hGH are SEQ ID NO 1 (hGH), and sequences 3 (muttRNA), and 16, 17 or 18 (TyrRS LW1, 5, or 6) described in Example 2 above.

[259] Once modified, the hGH variant comprising the carbonyl-containing amino acid is reacted with an aminooxy-containing PEG derivative of the form:

$R\text{-}PEG(N)\text{-}O\text{-}(CH_2)_n\text{-}O\text{-}NH_2$

where R is methyl, n is 3 and N is approximately 5,000 MW. The purified hGH containing *p*-acetylphenylalanine dissolved at 10 mg/mL in 25 mM MES (Sigma Chemical, St. Louis, MO) pH 6.0, 25 mM Hepes (Sigma Chemical, St. Louis, MO) pH 7.0, or in 10 mM Sodium Acetate (Sigma Chemical, St. Louis, MO) pH 4.5, is reacted with a 10 to 100-fold excess of aminooxy-containing PEG, and then stirred for 10 – 16 hours at room temperature (Jencks, W. *J. Am. Chem. Soc.* 1959, 81, pp 475). The PEG-hGH is then diluted into appropriate buffer for immediate purification and analysis.

Example 4

[260] Conjugation with a PEG consisting of a hydroxylamine group linked to the PEG via an amide linkage.

[261] A PEG reagent having the following structure is coupled to a ketone-containing non-naturally encoded amino acid using the procedure described in Example 3:

$R\text{-}PEG(N)\text{-}O\text{-}(CH_2)_2\text{-}NH\text{-}C(O)(CH_2)_n\text{-}O\text{-}NH_2$

where R = methyl, n=4 and N is approximately 20,000 MW. The reaction, purification, and analysis conditions are as described in Example 3.

Example 5

[262] This example details the introduction of two distinct non-naturally encoded amino acids into hGH

[263] This example demonstrates a method for the generation of an hGH polypeptide that incorporates non-naturally encoded amino acid comprising a ketone functionality at two positions among the following residues: E30, E74, Y103, K38, K41, K140, and K145. The hGH polypeptide is prepared as described in Examples 1 and 2, except that the suppressor codon is introduced at two distinct sites within the nucleic acid.

Example 6

[264] This example details conjugation of hGH polypeptide to a hydrazide-containing PEG and subsequent in situ reduction

[265] An hGH polypeptide incorporating a carbonyl-containing amino acid is prepared according to the procedure described in Examples 2 and 3. Once modified, a

hydrazide-containing PEG having the following structure is conjugated to the hGH polypeptide:

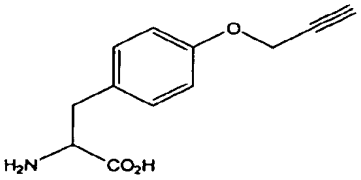
R-PEG(N)-O-$(CH_2)_2$-NH-C(O)$(CH_2)_n$-X-NH-NH_2

where R = methyl, n=2 and N = 10,000 MW and X is a carbonyl (C=O) group. The purified hGH containing *p*-acetylphenylalanine is dissolved at between 0.1-10 mg/mL in 25 mM MES (Sigma Chemical, St. Louis, MO) pH 6.0, 25 mM Hepes (Sigma Chemical, St. Louis, MO) pH 7.0, or in 10 mM Sodium Acetate (Sigma Chemical, St. Louis, MO) pH 4.5, isreacted with a 10 to 100-fold excess of aminooxy-containing PEG, and the corresponding hydrazone is reduced *in situ* by addition of stock 1M $NaCNBH_3$ (Sigma Chemical, St. Louis, MO), dissolved in H_2O, to a final concentration of 10-50 mM. Reactions are carried out in the dark at 4 °C to RT for 18-24 hours. Reactions are stopped by addition of 1 M Tris (Sigma Chemical, St. Louis, MO) at about pH 7.6 to a final Tris concentration of 50 mM or diluted into appropriate buffer for immediate purification.

Example 7

[266] This example details introduction of an alkyne-containing amino acid into hGH and derivatization with mPEG-azide.

[267] The following residues, 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155, are each substituted with the following non-naturally encoded amino acid:



[268] The sequences utilized for site-specific incorporation of p-propargyl-tyrosine into hGH are SEQ ID NO 1 (hGH), SEQ ID NO:4 (muttRNA), and 9, 10 or 11 described in Example 2 above. The hGH polypeptide containing the propargyl tyrosine is expressed in *E. coli* and purified using the conditions described in Example 3.

[269] The purified hGH containing propargyl-tyrosine dissolved at between 0.1-10 mg/mL in PB buffer (100 mM sodium phosphate, 0.15 M NaCl, pH = 8) and a 10 to 1000-fold excess of an azide-containing PEG is added to the reaction mixture. A catalytic amount of $CuSO_4$ and Cu wire are then added to the reaction mixture. After the mixture is incubated (e.g., about 4 hours at room temperature or 37° C, or overnight at 4 °C), H_2O is

added and the mixture is filtered through a dialysis membrane. The sample can be analyzed for the addition, e.g., by similar procedures described in Example 3.

[270] In this Example, the PEG will have the following structure:

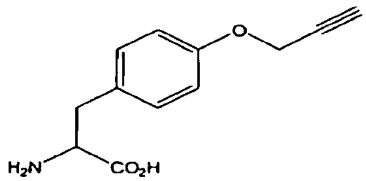
R-PEG(N)-O-$(CH_2)_2$-NH-C(O)$(CH_2)_n$-N_3

where R is methyl, n is 4 and N is 10,000 MW.

Example 8

[271] This example details substitution of a large, hydrophobic amino acid in hGH with propargyl tyrosine.

[272] A Phe, Trp or Tyr residue present within one the following regions of hGH: 1-5 (N-terminus); 32-46 (N-terminal end of the A-B loop); 97-105 (B-C loop); and 132-149 (C-D loop); and 184-191 (C-terminus), is substituted with the following non-naturally encoded amino acid as described in Example 3:



[273] Once modified, a PEG is attached to the hGH variant comprising the alkyne-containing amino acid. The PEG will have the following structure:

Me-PEG(N)-O-$(CH_2)_2$-N_3

and coupling procedures would follow those in Example 7. This will generate an hGH variant comprising a non-naturally encoded amino acid that is approximately isosteric with one of the naturally-occurring, large hydrophobic amino acids and which is modified with a PEG derivative at a distinct site within the polypeptide.

Example 9

[274] This example details generation of an hGH dimer separated by a PEG linker.

[275] The alkyne-containing hGH variant produced in Example 7 is reacted with a bifunctional PEG derivative of the form:

N_3-$(CH_2)_n$-C(O)-NH-$(CH_2)_2$-O-PEG(N)-O-$(CH_2)_2$-NH-C(O)-$(CH_2)_n$-N_3

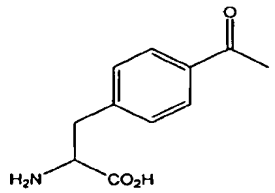
where n is 4 and the PEG has an average MW of approximately 5,000, to generate the corresponding hGH dimer where the two hGH molecules are physically separated by a high

molecular weight PEG. Coupling, purification, and analyses will be performed as in Examples 7 and 3.

Example 10

[276] This example details coupling of a saccharide moiety to hGH.

[277] One residue of the following is substituted with the non-natural encoded amino acid below: 29, 30, 33, 34, 35, 37, 39, 40, 49, 57, 59, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 107, 108, 111, 122, 126, 129, 130, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 159, 183, 186, and 187 as described in Example 3.

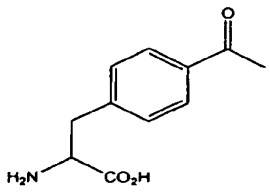


[278] Once modified, the hGH variant comprising the carbonyl-containing amino acid is reacted with a β-linked aminooxy analogue of N-acetylglucosamine (GlcNAc). The hGH variant (10 mg/mL) and the aminooxy saccharide (21mM) are mixed in aqueous 100mM sodium acetate buffer (pH 5.5) and incubated at 37° C for 7 to 26 h. A second saccharide is coupled to the first enzymatically by incubating the saccharide-conjugated hGH (5mg/mL) with UDP-galactose (16mM) and β-1,4-galacytosyltransferase (0.4 units/mL) in 150mM HEPES buffer (pH 7.4) for 48 h at ambient temperature (Schanbacher et al. *J. Biol. Chem.* 1970, 245, 5057-5061).

Example 11

[279] This example details generation of a PEGylated hGH antagonist.

[280] One of the following residues 1, 2, 3, 4, 5, 8, 9, 11, 12, 15, 16, 19, 22, 103, 109, 112, 113, (115), 116, 119, 120, 123, or 1277, is substituted with the following non-naturally encoded amino acid as described in Example 3.



[281] Once modified, the hGH variant comprising the carbonyl-containing amino acid will be reacted with an aminooxy-containing PEG derivative of the form:

$R\text{-}PEG(N)\text{-}O\text{-}(CH_2)_n\text{-}O\text{-}NH_2$

where R is methyl, n is 4 and N is 20,000 MW to generate an hGH antagonist comprising a non-naturally encoded amino acid that is modified with a PEG derivative at a single site within the polypeptide. Coupling, purification, and analyses is be performed as in Example 3.

Example 12

Generation of an hGH Dimer in which the hGH Molecules are Linked Directly

[282] An hGH variant comprising the alkyne-containing amino acid can be directly coupled to another hGH variant comprising the azido-containing amino acid, each of which comprise non-naturally encoded amino acid substitutions at the sites described in, but not limited to, Example 10. This will generate the corresponding hGH dimer where the two hGH variants are physically joined at the site 2 binding interface. Coupling, purification, and analyses is performed as in Examples 3, 6 and 7.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. An hGH polypeptide comprising a non-naturally encoded amino acid.

2. The hGH polypeptide of claim 1, wherein the hGH polypeptide is linked to a second hGH polypeptide.

3. The hGH polypeptide of claim 1, wherein the non-naturally encoded amino acid is linked to a water soluble polymer.

4. The hGH polypeptide of claim 3, wherein the water soluble polymer comprises a poly(ethylene glycol) moiety.

5. The hGH polypeptide of claim 4, wherein the poly(ethylene glycol) molecule is a bifunctional polymer.

6. The hGH polypeptide of claim 5, the bifunctional polymer is linked to a second polypeptide.

7. The hGH polypeptide of claim 6, wherein the second polypeptide is an hGH polypeptide.

8. The hGH polypeptide of claim 4, comprising at least two amino acids linked to a water soluble polymer comprising a poly(ethylene glycol) moiety.

9. The hGH polypeptide of claim 8, wherein at least one amino acid is a non-naturally encoded amino acid.

10. The hGH polypeptide of claim 4, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1-5, 32-46, 97-105, 132-149, and 184-191 from SEQ ID NO: 2.

11. The hGH polypeptide of claim 4, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1, 2, 3, 4, 5, 9, 11, 12, 15, 16, 19, 22, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,43, 44, 45, 46, 47, 48, 49, 52, 55, 57, 59, 65, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 109, 111, 112, 113, 115, 116, 119, 120, 122, 123, 126, 127, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147,

148, 149, 150, 151, 152, 153, 154, 155, 156, 158, 159, 161, 168, 172, 183, 184, 185, 186, 187, 188, 189, 190, 191 from SEQ ID NO: 2.

12. The hGH polypeptide of claim 11, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 29, 30, 33, 34, 35, 37, 39, 40, 49, 57, 59, 66, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 107, 108, 111, 122, 126, 129, 130, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 159, 183, 186, and 187, and a combination thereof from SEQ ID NO: 2.

13. The hGH polypeptide of claim 11, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 33, 35, 37, 57, 69, 70, 71, 74, 88, 91, 92, 94, 95, 98, 99, 101, 129, 131, 133, 134, 135, 136, 137, 140, 141, 142, 143, 145, 147, 154, 155, 156, 186, and 187, and a combination thereof from SEQ ID NO: 2.

14. The hGH polypeptide of claim 11, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 35, 88, 91, 92, 94, 95, 99, 101, 131, 133, 134, 135, 136, 140, 143, 145, and 155, and a combination thereof from SEQ ID NO: 2.

15. The hGH polypeptide of claim 4, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of 30, 74, 103, and a combination thereof, from SEQ ID NO: 2.

16. The hGH polypeptide of claim 1, wherein the hGH polypeptide comprises a substitution, addition or deletion that increases affinity of the hGH polypeptide for a growth hormone receptor.

17. The hGH polypeptide of claim 1, wherein the hGH polypeptide comprises a substitution, addition or deletion that increases the stability of the hGH polypeptide.

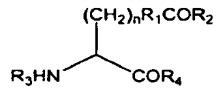
18. The hGH polypeptide of claim 16, comprising an amino acid substitution selected from the group consisting of F10A, F10H, F10I; M14W, M14Q, M14G; H18D; H21N; R167N; D171S; E174S; F176Y, I179T and combination thereof in SEQ ID NO: 2.

19. The hGH polypeptide of claim 1, wherein the non-naturally encoded amino acid is reactive toward a water soluble polymer that is otherwise unreactive toward any of the 20 common amino acids.

20. The hGH polypeptide of claim 1, wherein the non-naturally encoded amino acid comprises a carbonyl group, an aminooxy group, a hydrazine group, a hydrazide group, a semicarbazide group, an azide group, or an alkyne group.

21. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid comprises a carbonyl group.

22. The hGH polypeptide of claim 21, wherein the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, or substituted aryl; R_2 is H, an alkyl, aryl, substituted alkyl, and substituted aryl; and R_3 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_4 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

23. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid comprises an aminooxy group.

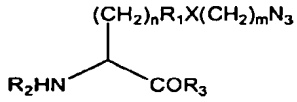
24. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid comprises a hydrazide group.

25. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid comprises a hydrazine group.

26. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid residue comprises a semicarbazide group.

27. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid residue comprises an azide group.

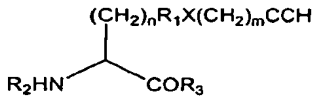
28. The hGH polypeptide of claim 27, wherein the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, substituted aryl or not present; X is O, N, S or not present; m is 0-10; R_2 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R_3 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

29. The hGH polypeptide of claim 20, wherein the non-naturally encoded amino acid comprises an alkyne group.

30. The hGH polypeptide of claim 29, wherein the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R_1 is an alkyl, aryl, substituted alkyl, or substituted aryl; X is O, N, S or not present; m is 0-10, R_2 represents one or more adjacent amino acids or zero adjacent amino acids in which case R_2 represents the amino terminus of a polypeptide and R_3 represents one or more adjacent amino acids or zero adjacent amino acids in which case R_3 represents the carboxyl terminus of a polypeptide.

31. The hGH polypeptide of claim 4, wherein the poly(ethylene glycol) molecule has a molecular weight of between about 1 and about 50 kDa.

32. The hGH polypeptide of claim 31, wherein the poly(ethylene glycol) molecule has a molecular weight of between 10 kDa and 40 kDa.

33. The hGH polypeptide of claim 4, which is made by reacting an hGH polypeptide comprising a carbonyl-containing amino acid with a poly(ethylene glycol) molecule comprising a hydroxylamine, hydrazine, hydrazide or semicarbazide group.

34. The hGH polypeptide of claim 33, wherein the hydroxylamine, hydrazine, hydrazide or semicarbazide group is linked to the poly(ethylene glycol) molecule through an amide linkage.

35 The hGH polypeptide of claim 4, which is made by reacting a poly(ethylene glycol) molecule comprising a carbonyl group with a polypeptide comprising a

non-naturally encoded amino acid that comprises a hydroxylamine, hydrazide or semicarbazide group.

36. The hGH polypeptide of claim 4, which is made by reacting an hGH polypeptide comprising an alkyne-containing amino acid with a poly(ethylene glycol) molecule comprising an azide moicty.

37. The hGH polypeptide of claim 4, which is made by reacting an hGH polypeptide comprising an azide-containing amino acid with a poly(ethylene glycol) molecule comprising an alkyne moiety.

38. Thc hGH polypeptide of claim 36 or claim 37, wherein the azide or alkyne group is linked to the poly(ethylene glycol) molecule through an amide linkage.

39. The hGH polypeptide of claim 4, wherein the poly(ethylene glycol) molecule is a branched polymer.

40. The hGH polypeptide of claim 39, wherein each branch of the poly(ethylene glycol) branched polymer has a molecular weight of between 5 kDa and 30 kDa.

41. The hGH polypeptide of claim 1, wherein the polypeptide is a growth hormone antagonist.

42. The hGH polypeptide of claim 41, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1, 2, 3, 4, 5, 8, 9, 11, 12, 15, 16, 19, 22, 103, 109, 112, 113, 115, 116, 119, 120, 123, or 127, and a combination thereof from SEQ ID NO: 2.

43. The hGH polypeptide of claim 41, wherein the non-naturally encoded amino acid is linked to a water soluble polymer.

44. The hGH polypeptide of claim 41, wherein the water soluble polymer comprises a poly(ethylene glycol) mioety.

45. The hGH polypeptide according to claim 41, wherein the non-naturally encoded amino acid linked to a water soluble polymer is present within the Site II region of the hGH polypeptide.

46. The hGH polypeptide according to claim 41, wherein the non-naturally encoded amino acid linked to a water soluble polymer prevents dimerization of the hGH receptor by preventing the hGH antagonist from binding to a second hGH receptor.

47. The hGH polypeptide according to claim 41, wherein an amino acid other than glycine is substituted for G120 in SEQ ID NO: 2.

48. The hGH polypeptide according to claim 47, wherein arginine is substituted for G120 in SEQ ID NO: 2.

49. The hGH polypeptide of claim 1, wherein the non-naturally encoded amino acid comprises a saccharide moiety.

50. The hGH polypeptide of claim 3, wherein the water soluble polymer is linked to the polypeptide via a saccharide moiety.

51. An isolated nucleic acid comprising a polynucleotide that hybridizes under stringent conditions to SEQ ID NO:1, wherein the polynucleotide comprises at least one selector codon and the polynucleotide encodes an hGH polypeptide of claim 1.

52. The isolated nucleic acid of claim 51, wherein the selector codon is selected from the group consisting of an amber codon, ochre codon, opal codon, and a four-base codon.

53. A method of making the hGH polypeptide of claim 4, the method comprising contacting an isolated hGH polypeptide comprising a non-naturally encoded amino acid with a water soluble polymer comprising a moiety that reacts with the non-naturally encoded amino acid.

54. The method of claim 53, wherein the water soluble polymer comprises a polyethylene glycol mioety.

55. The method of claim 53, wherein the non-naturally encoded amino acid residue comprises a carbonyl group, an aminooxy group, a hydrazide group, a semicarbazide group, an azide group, or an alkyne group.

56. The method of claim 55, wherein the non-naturally encoded amino acid residue comprises a carbonyl moiety and the water soluble polymer comprises a hydroxylamine, hydrazide or semicarbazide moiety.

57. The method of claim 55, wherein the non-naturally encoded amino acid residue comprises an alkyne moiety and the water soluble polymer comprises an azide moiety.

58. The method of claim 55, wherein the non-naturally encoded amino acid residue comprises an azide moiety and the water soluble polymer comprises an alkyne moiety.

59. The method of claim 54, wherein the polyethylene glycol moiety has an average molecular weight of between about 1 and about 50 kDa.

60. The method of claim 58, wherein the polyethylene glycol moiety is a branched polymer.

61. A composition comprising the hGH polypeptide of claim 1 and a pharmaceutically acceptable carrier.

62. The composition of claim 61, wherein the non-naturally encoded amino acid is linked to a water soluble polymer.

63. A method of treating a patient having a growth or development disorder comprising administering to the patient a therapeutically-effective amount of the pharmaceutical composition of claim 61.

64. A cell comprising a polynucleotide encoding the hGH polypeptide of claim 1.

65. The cell of claim 64, wherein the cell comprises an orthogonal RNA synthetase and orthogonal tRNA for substituting the non-naturally encoded amino acid into the hGH polypeptide.

66. A method of making an hGH polypeptide comprising a non-naturally encoded amino acid, the method comprising,

culturing cells comprising a polynucleotide or polynucleotides encoding an hGH polypeptide, an orthogonal RNA synthetase and an orthogonal tRNA under conditions to permit expression of the hGH polypeptide; and

purifying the hGH polypeptide from the cells.

67 . A method of increasing serum half-life or circulation time of hGH, the method comprising substituting a non-naturally encoded mino acid for any one or more amino acids in naturally occurring hGH.

68. An hGH polypeptide having a sequence shown in SEQ ID NO: 3, wherein at least one amino acid is substituted by a non-naturally encoded amino acid.

69. The hGH polypeptide of claim 68, wherein the non-naturally encoded amino acid is linked to a water soluble polymer.

70. The hGH polypeptide of claim 68, wherein the water soluble polymer comprises a poly(ethylene glycol) moiety.

71. The hGH polypeptide of claim 68, wherein the non-naturally encoded amino acid is substituted at a position selected from the group consisting of residues 1-5, 82-90, 117-134, and 169-176 from SEQ ID NO: 3.

72. The hGH polypeptide of claim 68, wherein the non-naturally encoded amino acid comprises a carbonyl group, an aminooxy group, a hydrazide group, a hydrazine group, a semicarbazide group, an azide group, or an alkyne group.

73. The hGH polypeptide of claim 70, wherein the poly(ethylene glycol) moiety has a molecular weight of between about 1 and about 50 kDa.

74. The hGH polypeptide of claim 70, wherein the poly(ethylene glycol) moiety has a molecular weight of between 5 kDa and 40 kDa.

75. The hGH polypeptide of claim 70, wherein the polyethylene glycol moiety is a branched polymer.

76. A pharmaceutical composition comprising the hGH polypeptide of claim 68 and a pharmaceutically acceptable carrier.

ABSTRACT OF THE DISCLOSURE

Modified Human Growth Hormone Polypeptides and Their Uses

Modified human Growth Hormone (hGH) polypeptides and uses thereof are provided.

SEQUENCE LISTING

SEQ ID #	Sequence	*Notes*	Protein f tRNA or RS
1	MATGSRTSLLLAFGLLCLPWLQEGSAFPTIPLSRLFDNAMLRAHRLHQLAFD TYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLL LIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSP RTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRS VEGSCGF	*Full-length amino acid sequence of hGH*	protein
2	FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLC FSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASD SNVYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKN YGLLYCFRKDMDKVETFLRIVQCRSVEGSCGF	*The mature amino acid sequence of hGH (isoform 1)*	protein
3	FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFNPQTSLCFSESIPTPSNREETQQ KSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQ TLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDK VETFLRIVQCRSVEGSCGF	*The 20-kDa hGH variant in which residues 32-46 of hGH are deleted*	protein
4	CCGGCGGTAGTTCAGCAGGGCAGAACGGCGGACTCTAAATCCGCATGGC GCTGGTTCAAATCCGGCCCGCCGGACCA	*M. jannaschii* $mtRNA^{Tyr}_{CUA}$	tRNA
5	CCCAGGGTAG CCAAGCTCGG CCAACGGCGAC GGACTCTAA ATCCGTTCTC GTAGGAGTTC GAGGGTTCGA ATCCCTTCCC TGGGACCA	*HLAD03; an optimized amber supressor tRNA*	tRNA
6	GCGAGGGTAG CCAAGCTCGG CCAACGGCGA CGGACTTCCT AATCCGTTCT CGTAGGAGTT CGAGGGTTCG AATCCCTCCC CTCGCACCA	*HL325A; an optimized AGGA frameshift supressor tRNA*	tRNA
7	MDEFEMIKRNTSEIISEEELREVLKKDEKSAGIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS TFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNT YYYLGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-L-phenylalanine* *p-Az-PheRS(6)*	RS
8	MDEFEMIKRNTSEIISEEELREVLKKDEKSAGIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS SFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNT SHYLGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-benzoyl-L-phenylalanine* *p-BpaRS(1)*	RS
9	MDEFE MIKRN TSEII SEEEL REVLK KDEKS AAIGF EPSGK IHLGH YLQIK KMIDL QNAGF DIIIL LADLH AYLNQ KGELD EIRKI GDYNK KVFEA MGLKA KYVYG SPFQL DKDYT LNVYR LALKT TLKRA RRSME LIARE DENPK VAEVI YPIMQ VNAIY LAVD VAVGG MEQRK IHMLA RELLP KKVVC IHNPV LTGLD GEGKM SSSKG NFIAV DDSPE EIRAK IKKAY CPAGV VEGNP IMEIA KYFLE YPLTI KRPEK FGGDL TVNSY EELES LFKNK ELHPM DLKNA VAEEL IKILE PIRKR L	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS
10	MDEFE MIKRN TSEII SEEEL REVLK KDEKS AAIGF EPSGK IHLGH YLQIK KMIDL QNAGF DIIIL LADLH AYLNQ KGELD EIRKI GDYNK KVFEA MGLKA KYVYG SPFQL DKDYT LNVYR LALKT TLKRA RRSME LIARE DENPK VAEVI YPIMQ VNIPY LPVD VAVGG MEQRK IHMLA RELLP KKVVC IHNPV LTGLD GEGKM SSSKG NFIAV DDSPE EIRAK IKKAY CPAGV VEGNP IMEIA KYFLE YPLTI KRPEK FGGDL TVNSY EELES LFKNK ELHPM DLKNA VAEEL IKILE PIRKR L	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS
11	MDEFE MIKRN TSEII SEEEL REVLK KDEKS AAIGF EPSGK IHLGH YLQIK KMIDL QNAGF DIIIL LADLH AYLNQ KGELD EIRKI GDYNK KVFEA MGLKA KYVYG SKFQL DKDYT LNVYR LALKT TLKRA RRSME LIARE DENPK VAEVI YPIMQ VNAIY LAVD VAVGG MEQRK IHMLA RELLP KKVVC IHNPV LTGLD GEGKM SSSKG NFIAV DDSPE EIRAK IKKAY CPAGV VEGNP IMEIA KYFLE YPLTI KRPEK FGGDL TVNSY EELES LFKNK ELHPM DLKNA VAEEL IKILE PIRKR L	*Aminoacyl tRNA synthetase for the incorporation of propargyl-phenylalanine* *Propargyl-PheRS*	RS

SEQ ID #	Sequence	*Notes*	Protein of tRNA or RS
12	MDEFEMIKRNTSEIISEEELREVLKKDEKSATIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS NFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVN PLHYQGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSS SKGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(1)*	RS
13	MDEFEMIKRNTSEIISEEELREVLKKDEKSATIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS SFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNP LHYQGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(3)*	RS
14	MDEFEMIKRNTSEIISEEELREVLKKDEKSALIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS TFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNP VHYQGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(4)*	RS
15	MDEFEMIKRNTSEIISEEELREVLKKDEKSATIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS SFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNP SHYQGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine* *p-Az-PheRS(2)*	RS
16	MDEFEMIKRNTSEIISEEELREVLKKDEKSALIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS EFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVN GCHYRGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSS SKGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW1)*	RS
17	MDEFEMIKRNTSEIISEEELREVLKKDEKSALIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS EFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVN GTHYRGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSS SKGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW5)*	RS
18	MDEFEMIKRNTSEIISEEELREVLKKDEKSAAIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS EFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVN GGHYLGVDVIVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (LW6)*	RS
19	MDEFEMIKRNTSEIISEEELREVLKKDEKSAAIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS RFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVN VIHYDGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSS SKGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (AzPheRS-5)*	RS
20	MDEFEMIKRNTSEIISEEELREVLKKDEKSAGIGFEPSGKIHLGHYLQIKKMID LQNAGFDIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGS TFQLDKDYTLNVYRLALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNT YYYLGVDVAVGGMEQRKIHMLARELLPKKVVCIHNPVLTGLDGEGKMSSS KGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNPIMEIAKYFLEYPLTIKRPEKF GGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILEPIRKRL	*Aminoacyl tRNA synthetase for the incorporation of p-azido-phenylalanine (AzPheRS-6)*	RS

Application Data Sheet

Application Information

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Suggested Group Art Unit::	
Sequence Submission::	
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Number of copies of CRF::	
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Attorney Docket Number::	021862-000400US
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Request for Non-Publication::	No
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Total Drawing Sheets::	0
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Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
Secrecy Order in Parent Appl.::	No

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Correspondence Customer Number:: 20350

Representative Information

Representative Customer Number:: 20350

Domestic Priority Information

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Foreign Priority Information

Country:: Application number:: Filing Date::

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